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Production of poly-B-hydroxybutyrate in the Azotobacteraceae

Anthony Glenn Ostle
Iowa State University

Ostle, Anthony Glenn

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Anthony Glenn Ostle

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INTRODUCTION

Poly-β-hydroxybutyrate (PHB) is a bacterial storage compound which has several unique properties. This compound, which may comprise over 80% of the dry weight of the cell (Schlegel and Gottschalk, 1962), has a melting point of 160-172° C (Dawes, 1975) and is insoluble in water and many common solvents (Akita et al., 1976). Because of its unique properties, PHB has been proposed as a source of plastics (Baptist and Werber, 1965) and as a petrochemical substitute (Young, 1975).

Because of the large size of the PHB molecule and the stereospecificity of its subunits, efforts to artificially synthesize PHB have been expensive and not well suited to large-scale PHB production (Marchessault and Faure, 1974).

The genus Azotobacter is known to produce PHB in large quantities from simple, inexpensive substrates while fixing atmospheric dinitrogen (Dawes, 1975). The conditions for the production of large amounts of PHB in Azotobacter appear to be simpler to implement and control than those of other organisms proposed for the mass biosynthesis of PHB (Baptist, 1962; Dawes, 1975). Azotobacter is believed to accumulate PHB only when fixing atmospheric dinitrogen, and does not accumulate the polymer when amino acids or ammonium salts are supplied as nitrogen sources (Dawes, 1975).

PHB may be isolated from cells by any of several well-known methods, most of which are modifications of a hypochlorite digestion method

developed by Williamson and Wilkinson (1958). PHB is assayed by degrading the polymer to crotonate and determining the ultraviolet spectrum of this breakdown product (Law and Slepecky, 1961), or by staining the intact granules with Sudan Black B (Burdon, 1946). The Sudan Black B stain is not notably sensitive.

Before PHB can be of use as a chemical feedstock, a plastic, or otherwise be of commercial value, methods for its mass biosynthesis must be determined. Further, the connection between PHB accumulation and nitrogen fixation in Azotobacter is of interest in understanding the physiology of nitrogen fixation, which is increasingly the focus of worldwide interest. The methods for the detection and assay of PHB are also in need of improvement.

This research has attempted to determine the conditions necessary to produce PHB in large quantities in Azotobacter. Strains of Azotobacter accumulating PHB under various conditions have been isolated or produced by genetic modification. The optimum conditions for the production of PHB in these strains have been determined. A fermentation scheme resulting in more PHB per unit of production medium than other published means has been developed.

The staining of PHB has been investigated, and a stain for PHB superior to the traditional Sudan Black B stain has been reported. Further, methods for the recovery of PHB from producer cells have been optimized.

The relationship between nitrogen and PHB accumulation in Azotobacter has been investigated, and the ability of this organism to accumulate PHB in large quantities when a fixed nitrogen source is supplied has been documented. The relationship between nitrogen and PHB accumulation in several nitrogenase mutants of Azotobacter has been investigated, shedding new light on the connection between PHB and dinitrogen fixation in this genus.

The reported effect of several medium supplements on PHB accumulation in Azotobacter has been investigated and disproven.

The relationship between nitrogen source, PHB accumulation, and the process of encystment in Azotobacter has been investigated, and the prerequisites for encystment in this genus have been modified as a result of these studies. The importance of PHB accumulation in the encystment process has been examined by the use of nitrogenase mutants and various nitrogen sources.

LITERATURE REVIEW

Definition

Poly-β-hydroxybutyrate (PHB) is a bacterial polymeric ester which functions as an energy and/or carbon reserve in the cell. The compound was first recognized by Lemoigne (1926) who realized that PHB was likely the compound responsible for many of the lipid inclusions found in bacterial cells. Earlier, nonspecific references to PHB in bacteria date to the turn of the century (Beijerinck, 1901). These lipid inclusions, which may be recognized for their affinity for the dye Sudan Black B (Lemoigne et al., 1949) or by staining with Nile Blue Sulfate (Ostle and Holt, 1982), have frequently been identified as containing PHB (Lemoigne et al., 1949; Weibull, 1953). The PHB polymer (Figure 1) was analyzed by Williamson and Wilkinson (1958) who reported a molecular weight of about 5000 daltons and a chain length of ca. 60 residues ($C_4H_6O_2$) when the polymer was isolated by hypochlorite digestion.

Other researchers (Alper et al., 1963) have reported molecular weights ranging from 10,000 daltons to 128,000 daltons (by the Archibald method of ultracentrifugation) depending upon the extraction methods used. The higher molecular weights correspond to ca. 2,500 residues.

The role of PHB as an energy/carbon storage compound was elucidated by Macrae and Wilkinson (1958) and by Doudoroff and Stanier (1957). The amount of PHB found in a cell varies with the cell type, age, and cultural conditions. PHB concentrations as high as 80% of cell dry

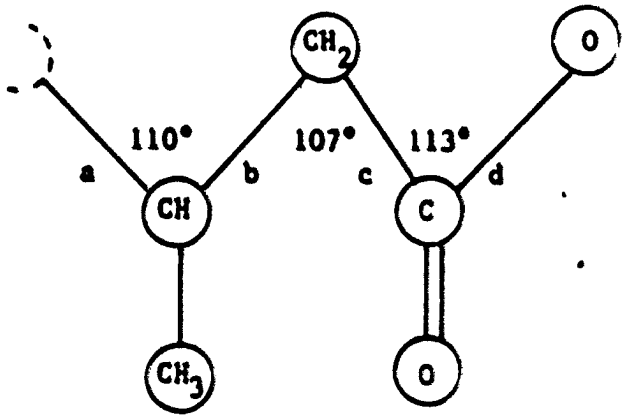

FIGURE 1. Poly-β-hydroxybutyrate (From Akita et al., 1976)

weight have been reported (Schlegel and Gottschalk, 1962).

Occurrence

Poly-β-hydroxybutyrate has been found in a variety of bacterial cells. To date, PHB has not been discovered in any eukaryotic organisms,[1] or in the rickettsias. A list of the organisms in which PHB has been reported may be found in Table 1. For each organism, a reference is provided, although this is not necessarily the original report of PHB in the organism.

It may be noted that PHB is most frequently found in free-living organisms and is virtually absent in pathogens. This is to be expected,

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[1] There is one report of PHB in a yeast, apparently unconfirmed (Nuti and Lepidi, 1974).

TABLE 1. Bacteria possessing poly-ß-hydroxybutyrate

Asticcacaulis (Poindexter, 1974a)

Azotobacter agilis (Stockdale et al., 1965)

Azotobacter beijerinckii (Jackson and Dawes, 1976)

Azotobacter chroococcum (Nuti et al., 1972)

Azotobacter vinelandii (Dawes and Ribbons, 1964)

Bacillus anthracis (Dawes and Ribbons, 1974)

Bacillus cereus (Macrae and Wilkinson, 1958)

Bacillus megaterium (Macrae and Wilkinson, 1958)

Bacillus sphericus (Slayter, 1970)

Beggiatoa (Lesdbetter, 1974)

Beijerinckia (Becking, 1974)

Caulobacter (Poindexter, 1974b)

Chlorogloea fritschii (Jansen and Sicko, 1971, 1973)

Chromatium (Pfennig and Truper, 1974)

Chromobacterium (Forsyth et al., 1958)

Clostridium botulinum (Emeruwa and Hawirko, 1973)

Ferrobacillus ferrooxidans (Wang and Lundgren, 1969)

Hydrogenomonas eutropha (Oeding and Schlegel, 1973)

Lampropedia (Seeley, 1974)

Micrococcus halodenitrificans (Sierra and Gibbons, 1962a,b)

Table 1. (Continued)

Nitrobacter winogradski (VanGool et al., 1969)
Paracoccus (Doudoroff, 1974)
Pseudomonas spp. (Morris and Roberts, 1959, Levine and Woolochow, 1960)
Pseudomonas mallei (Worley and Young, 1945)
Rhizobium (Gerson et al., 1978)
Rhodospirillaceae (Pfennig and Truper, 1974)
Rhodospirillum rubrum (Stenier et al., 1959, Moskowitz and Merrick, 1969)
Sphaerotilus natans (Dawes and Ribbons, 1964, Rouf and Stokes, 1962)
Spirillum anulus (Dawes and Ribbons, 1964)
Spirillum itersonii (Dawes and Ribbons, 1964)
Spirillum serpens (Dawes and Ribbons, 1964)
Streptomyces aureofaciens (Kannan and Rehack, 1970)
Streptomyces griseus (Kannan and Rehack, 1970)
Streptomyces nouresi (Kannan and Rehack, 1970)
Steptomyces olivaceus (Kannan and Rehack, 1970)
Zoogloea spp. (Crabtree and McCoy, 1974)
Zoogloea ramigera (Fukui et al., 1976, Tomita and Saito, 1976)

as microorganisms synthesize storage compounds when faced with starvation (Chen and Alexander, 1972). Free-living microbes are more

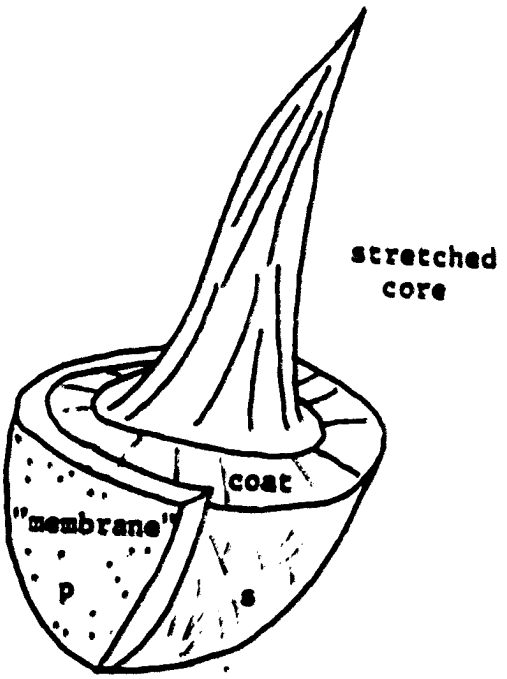
likely to face starvation than are pathogens or symbiotes which exist in the homeostatic environment of the host organism. Calow and Jennings (1977) found that storage compounds are synthesized in response to a decrease in nutrient level indicative of future starvation. However, several studies with PHB have shown that the polymer is actively synthesized during the exponential growth phase of the organism and decreased in quantity during the stationary phase (Law and Slepecky, 1961; Alper et al., 1963; Akita et al., 1976). It may be that the gradual decline of nutrient concentration during exponential growth triggers the production of PHB in these organisms. A notable exception to the limitation of PHB to free-living organisms is the genus Rhizobium (Gerson et al., 1978). However, rhizobia are known to exist independently of their plant host, and its production of PHB may be advantageous in this free-living state.

It may also be noted that the majority of the organisms in which PHB has been reported are rather large rods or filamentous organisms. Since the original detection of PHB in a microbe is usually by staining of the PHB inclusions with Sudan Black B, it may be that the smaller rods or cocci would not contain inclusions large enough to be easily seen.

PHB exists as discrete inclusions or granules in a cell. The morphology of these granules has been investigated by Merrick and Doudoroff (1964) and by Ellar et al. (1968). Dunlop and Robards (1973) made a detailed examination of the PHB granules of Bacillus cereus, examining freeze-etched preparations with electron microscopy. They

found that the granules had a diameter of 240-720 nm in intact cells. A central core of 140-370 nm which remained viscous during the freeze-fracture procedure was noted. When cells were prepared by the freeze-etch technique or by carbon replica methods, a structure which appeared to be a membrane was seen surrounding the granules. This structure, which did not appear to be a unit membrane, was not seen in intact, thin sectioned cells. These granules have been reported to be associated with the cell membrane (Pfister et al., 1969). Although Dunlop and Robards reported the central core as a distinct structure, Sleytr (1970) believed that the stretching of the PHB granules was an artifact of preparation due to the sudden expansion of a compressed portion of the granule. Dunlop and Robards reported that the area around the stretched core did not expand upon freeze-fracture, and named this area the "coat". A representation of a PHB granule according to Dunlop and Robards is presented in Figure 2.

Ellar et al. (1968) reported that when PHB granules were disrupted by dilute acetone, 10-15 nm fibrils and 5 nm filaments were produced. It is not known if this indicates a subunit structure in the granules. If a subunit structure is present, it is resistant to many treatments, including sonication, hypochlorite digestion, and lysozyme (Weibull, 1953).



p = particulate surface of specimens treated by sonication
s = stranded surface of hypochlorite extracted specimens

FIGURE 2. PHB granule (from Dunlop and Robards, 1973)

Metabolism

Anabolism

Doudoroff and Stanier (1957) reported that PHB is the primary product of carbon assimilation in Pseudomonas saccharophila and Rhodospirillum rubrum with 60-90% of the assimilated carbon found in PHB (assayed via

^{14}C-labeled acetate and butyrate). When external carbon sources were removed, PHB catabolism was rapid, with 90% of the polymer being degraded in 12 hours. Since ^{14}C activity remained in the cell after PHB catabolism, Doudoroff and Stanier concluded that PHB served as a carbon source for the cell. These authors also identified acetate as a probable precursor of PHB.

The endogenous metabolism of PHB was reviewed by Dawes and Ribbons (1964). These authors summarized earlier work by Merrick and Doudoroff (1961), and Haward et al. (1959) and proposed the following synthetic pathway for PHB (Figure 3). This work was based on the incorporation of ^{14}C-β-hydroxybutyryl-CoA into PHB (Merrick and Doudoroff, 1961).

The pathway in Figure 3 was modified by Moskowitz and Merrick (1969) to include crotonyl-CoA as a precursor of D-(-)-β-hydroxybutyryl-CoA.

The enzymes responsible for the reactions in Figure 3 were discussed by Senior and Dawes (1970). β-ketothiolase condenses two acetyl-CoA molecules to acetoacetyl-CoA + CoA. Acetoacetyl-CoA reductase reduces acetoacetyl-CoA to β-hydroxybutyryl-CoA. Polyhydroxybutyrate polymerase (poly-β-hydroxybutyrate synthetase) then forms PHB from β-hydroxybutyryl-CoA. An acyl carrier protein is not involved.

The studies of Senior and Dawes were performed with Azotobacter beijerinckii with concurrent nitrogen fixation. It was noted that the reoxidation of NADPH or NADH might compete with the reducing power needed for nitrogen fixation. It was also noted that acetoacetyl-CoA

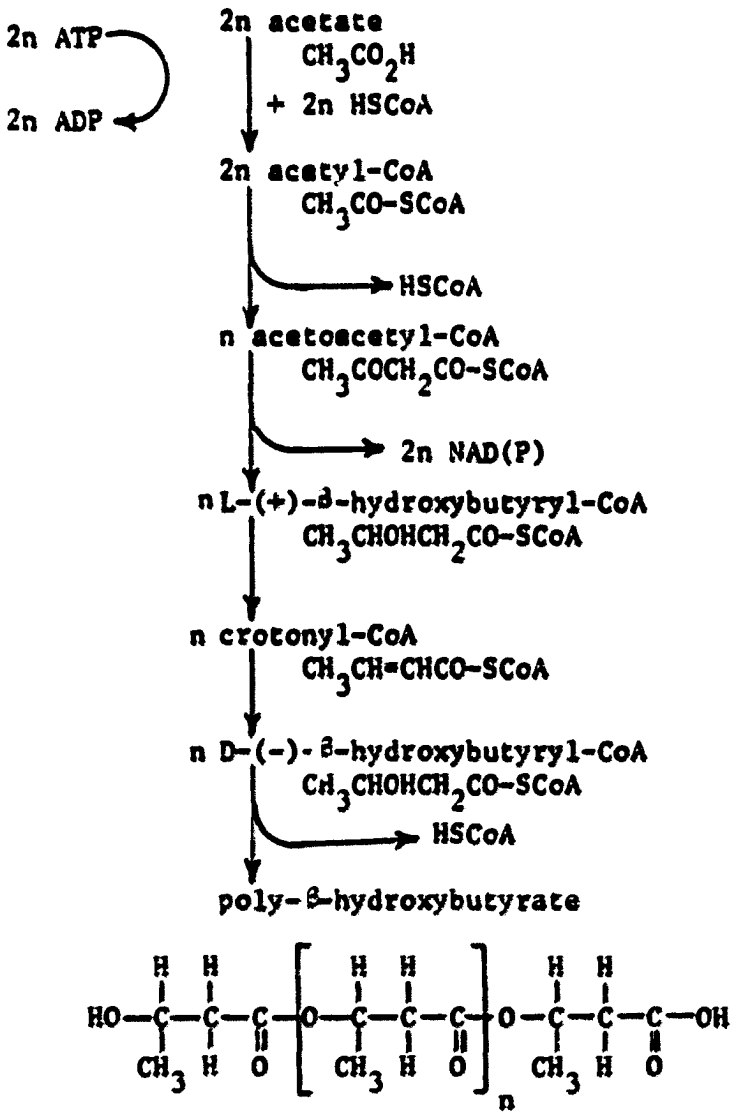


FIGURE 3. Synthesis of PHB (from Dawes and Ribbons, 1964)

reductase was able to utilize NADPH at five times the rate obtained with NADH in the reductive step. It is believed that glyceraldehyde 3-phosphate reacts with NAD(P)H in these cells to provide reducing power for PHB synthesis (Senior and Dawes, 1970).

Further studies (Senior and Dawes, 1971a) revealed that PHB production in Azotobacter was regulated by the intracellular $NAD(P)H/NAD(P)^+$ ratio. Since, under oxygen limitation, reduced coenzyme accumulates, anabolism and catabolism (in the TCA cycle) are curtailed. This is due to inhibition of glucose 6-phosphate dehydrogenase by NADH and NADPH. Isocitrate dehydrogenase and citrate synthetase are similarly affected. The fixation of nitrogen by the cell is also inhibited by the decrease in reducing power available for this purpose. Under these conditions, synthesis of PHB acts as an electron sink, reoxidizing NAD(P)H and restoring the above cell functions (Senior and Dawes, 1971a, 1971b). The synthesis of PHB in Azotobacter was found to be affected by limitations on the available carbon, oxygen, and nitrogen (Senior et al., 1972; Peterson and Hsu, 1977; Ward et al., 1977). Senior et al. (1972) noted that if Azotobacter was nitrogen and oxygen limited in a chemostat, PHB accumulated (up to 74% of cell dry weight in batch cultures and 50% in chemostats). When only oxygen was limited, little PHB accumulated. When nitrogen was limited, excess reducing power was channeled into PHB synthesis, with PHB serving as an electron sink. Ward et al. (1977) confirmed these observations, and noted that limiting available carbon lowers the PHB content of Azotobacter.

Ruhr and Schlegel (1975) investigated the condensation of two acetyl-CoA molecules to acetoacetyl-CoA plus CoA by β-ketothiolase and noted that this enzyme was further controlled by product inhibition. β-ketothiolase was 95% inhibited by 0.5 mM CoA.

PHB synthesis in Clostridium botulinum was investigated by Emeruwa and Hawirko (1973), and the role of ^{14}C acetate as a PHB precursor was confirmed. ^{14}C-labeled butyric acid was not a PHB precursor.

The diversity of PHB-producing organisms leads to variations in the basic synthetic pathway presented in Figure 3. Moskowitz and Merrick (1969) investigated PHB synthesis in Rhodospirillum rubrum and isolated two enoyl hydrases from this organism. These enzymes functioned in the hydration of crotonyl-CoA to β-hydroxybutyryl-CoA. One enzyme produced the D-(-) isomer of β-hydroxybutyryl-CoA while the other enzyme was specific for the L-(+) isomer. The combined action of these enzymes resulted in the racemization of D-(-)-β-hydroxybutyryl-CoA to L-(+)-β-hydroxybutyryl-CoA. Crotonyl-CoA was believed to be the intermediate in this racemization (see Figure 3).

PHB synthesis in the hydrogen-oxidizing bacterium Hydrogenomonas was studied by Schlegel et al. (1961a,b) and a crotonyl-CoA intermediate was also postulated. Both β-hydroxybutyric acid and crotonate were able to serve as substrates. It was postulated that crotonate is enzymatically transformed into crotonyl-CoA or β-hydroxybutyrate, either of which could then enter the basic synthetic pathway at β-hydroxybutyryl-CoA (Schlegel et al., 1961a,b; Dawes and Ribbons, 1964). Utilization of

β-hydroxybutyrate could not be demonstrated in Rhodospirillum rubrum (Dawes and Ribbons, 1964).

An anaerobic, light-dependent pathway for PHB synthesis in Rhodospirillum rubrum was proposed by Stanier et al. (1959) and may be summarized as follows:

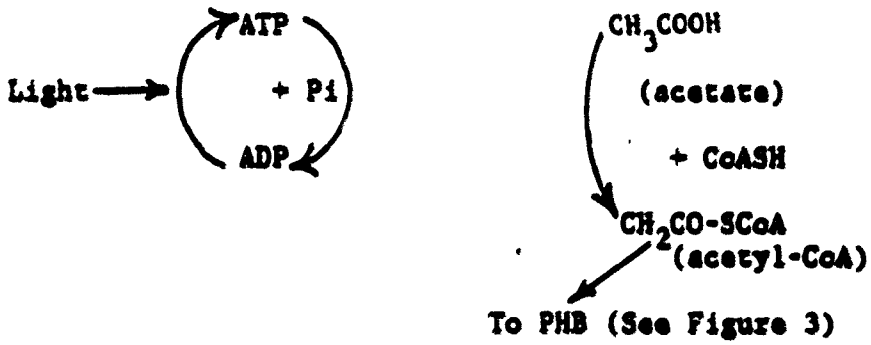


FIGURE 4. Light dependent PHB synthesis in Rhodospirillum rubrum (Stanier et al., 1959).

In the light-dependent scheme, reducing power for the conversion of acetoacetyl-CoA to β-hydroxybutyryl-CoA may be provided either by electrochemical coupling through a photochemically generated reductant or by NAD(P)H generated enzymatically from molecular hydrogen, but this has not been completely resolved (Stanier et al., 1959).

The location of the PHB-synthetase enzymes is now the subject of some controversy. It is generally accepted that the enzyme(s) are associated with the PHB granules, and are dependent upon them for activity. Greibel and Merrick (1971) extracted PHB granules from Bacillus

megaterium KM by mild alkaline extraction (0.01 M NaOH) which solubilized a protein fraction associated with the granules. This fraction was separated by chromatography (on Bio-gel A-15M) into two fractions, one of which, when combined with the extracted granules, showed PHB synthetase activity. Either fraction would protect the granule from enzymatic degradation, and could be inactivated by trypsin, alkaline extraction, or modified by an activator.

The protein fraction with PHB-synthetase activity was capable of forming PHB from D-(-)-β-hydroxybutyryl-CoA when the enzyme was associated with preexisting granules. The enzyme was quite labile and required special buffer systems to preserve activity. The optimum pH of the enzyme was 7.5, and a KM of 3.2×10^{-4} M (by Linewaver-Burk plot) was noted. Greibel and Merrick proposed the following pathwayfor the enzyme (where A-I is the enzymatically active protein fraction, Figure 5).

The acyl enzyme intermediate could not be isolated without the presence of PHB granules. The proposed pathway apparently contradicts the assertion of Senior and Dawes (1970) that an acyl carrier protein was not involved in PHB synthesis. Fukui et al. (1976) reported the isolation of soluble PHB synthetase from Zoogloea ramigera along with a PHB synthetase associated with PHB granules. Fukui et al. were uncertain whether or not these two enzymes were in fact identical. Although the two enzymes had the same substrate specificity and pH optimum (D-(-)-β-hydroxybutyryl-CoA and pH 7.0) soluble enzyme could not be generated from particulate enzyme. The soluble enzyme was capable of

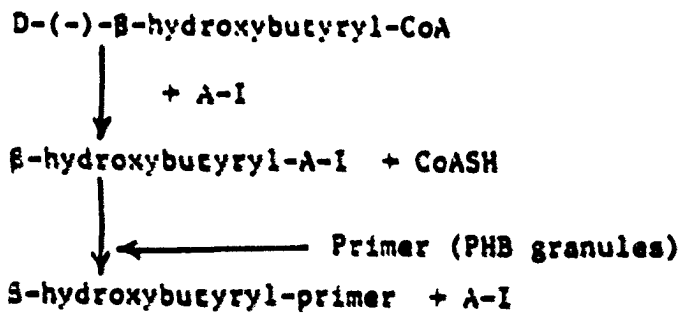


FIGURE 5. Synthetic pathway of PHB-polymerase (Greibel and Merrick, 1971).

synthesizing PHB from D-(-)-β-hydroxybutyryl-CoA without the presence of preexisting PHB as a primer. After granules were formed, the majority of PHB synthetase activity was associated with the granules as particulate enzyme (Fukui et al., 1976).

Greibel and Merrick (1971) believed that PHB granules were surrounded by a membrane in which the synthetase activity was located. This is supported by the electron microscopy of Dunlop and Robards (1973) who reported a membrane around thin-sectioned PHB granules which was not a unit membrane. Nuti et al. (1972) also reported membranes around PHB granules although they could not be seen in the published electron micrographs. However, the ability of the granule-associated protein fractions to reassociate with the granules after removal independently of the other protein fractions and to retain enzymatic activity without the other fractions would seem to weigh against the presence of a

membrane. Further, no reports of lipopolysaccharides[2] or other macromolecules often associated with bacterial membranes have been made in connection with the postulated granular membrane. It is possible that what has been identified as a membrane is actually PHB associated protein with enzymatic and regulatory functions, in which case, membrane would seem a poor term (Greibel and Merrick, 1971; Dunlop and Robards, 1973; Fukui et al., 1976).

Catabolism

Catabolism of PHB appears to be intimately associated with the PHB granules (Merrick and Doudoroff, 1961) and often with associated cell processes requiring large amounts of energy and carbon supplied by PHB. Thus, Kannan and Rehack (1970) reported that PHB accumulated by Streptomyces spp. was used for the production of antibiotics (e.g. streptomycin) and for sporulation. Lin and Fong (1977) noted that the production of cysts by Azotobacter vinelandii was associated with the rapid metabolism of the cell's PHB reserves and that PHB contained in the cysts was used for outgrowth. The role of PHB metabolism in the encystment cycle of Azotobacter has been reviewed by Sadoff (1975). The role of PHB in the formation of bacterial endospores has been established in Bacillus and Clostridium spp. PHB is necessary for endospore formation in Bacillus megaterium, disappearing during spore

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[2] A lipid content of 0.5% in native PHB granules has been reported (Greibel et al., 1968).

formation. Asporogenous strains of Bacillus megaterium did not metabolize PHB although the polymer was accumulated (Williamson and Wilkinson, 1958; Slepecky and Law, 1960a). The accumulation of PHB in some strains of Bacillus appears to be linked to the early events in the sporulation cycle, perhaps being triggered by nutrient depletion but not reversible once triggered (J. F. Charba, Dept. of Biology, University of Central Florida, personal communication, 1981). The metabolism of PHB in Clostridium appears to be related to that in Bacillus, in both sporulating and asporogenous strains (Emeruwa and Hawirko, 1973). Approximately 2% (by weight) PHB was noted in the endospores of Clostridium, suggesting a possible role in the outgrowth of spores (Emeruwa and Hawirko, 1973).

The pathway in Figure 6 was suggested by Dawes and Ribbons (1964) for the catabolism of PHB.

PHB depolymerase (esterase) was demonstrated by Merrick and Doudoroff (1961) in Rhodospirillum rubrum. Greibel and Merrick (1971) found that PHB from Bacillus megaterium KM could be degraded by Rhodospirillum rubrum depolymerase and by an extracellular depolymerase from Pseudomonas lemoignei. The extracellular depolymerase was found to hydrolyze PHB to dimers and trimers as well as to β-hydroxybutyrate.

Greibel and Merrick (1971) found that the second protein extract from PHB granules (the first being a PHB polymerase) had a regulatory effect on depolymerization. This A-II fraction was a potent inhibitor of PHB hydrolysis when associated with PHB granules. The A-II protein could be

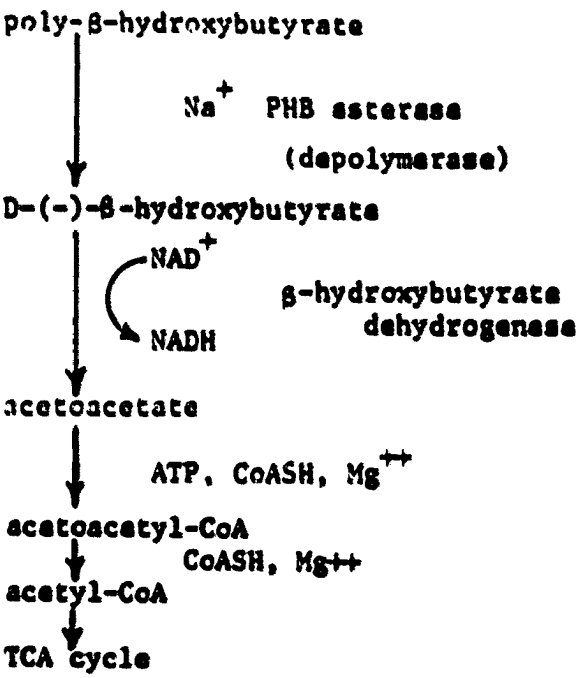


FIGURE 6. Catabolism of PHB (Dawes and Ribbons, 1964).

destroyed by trypsin or rendered ineffective by an activator whose mechanism was unknown. The PHB polymerase also had a protective effect when associated with PHB granules. When these proteins were removed or an activator was present, the polymer was rapidly degraded by PHB depolymerase. It has also been reported that granules isolated by alkaline hypochlorite methods are not susceptible to enzymatic degradation, presumably due to the destruction of the "membrane" (Greibel and Merrick, 1971; Barber and Nakata, 1973).

Dawes (1975) has proposed that the depolymerase of Azotobacter beijerinckii might bind to a specific site on the granule and that the other proteins bound to the granule (polymerase etc.) might inhibit the binding or activity of the soluble depolymerase.

The D-(-)-β-hydroxybutyrate dehydrogenase shown in Figure 6 has been isolated from many organisms known to utilize PHB. This enzyme has been reported to have a pH optimum of 8.0 to 8.5 and is competitively inhibited by NADH (not NADPH) and by pyruvate and 2-oxoglutarate (Dawes, 1975).

The catabolic pathway for PHB in A. beijerinckii has been modified by Dawes (1975) to include an acetoacetate-succinate-CoA CoA transferase discovered in this organism. This pathway may also exist in Hydrogenomonas (Oeding and Schlegel, 1973).

By the control mechanisms in Figure 7, PHB catabolism should be favored by a high NAD^+ concentration and a low pyruvate concentration. Lack of an exogenous carbon source would produce a low pyruvate concentration via a lowered glycolysis rate (Dawes, 1975).

It is of interest to note that PHB depolymerase has been found in some fungi, and that these fungi are also capable of degrading synthetic polyesters. It is postulated that the PHB depolymerase is responsible for this activity (Tanaka et al., 1976a,b,c).

Isolation of PHB

Extraction of PHB is usually performed by either sodium hypochlorite digestion of the majority of the cell, leaving PHB, or by extraction of the polymer with neutral solvents, usually chloroform.

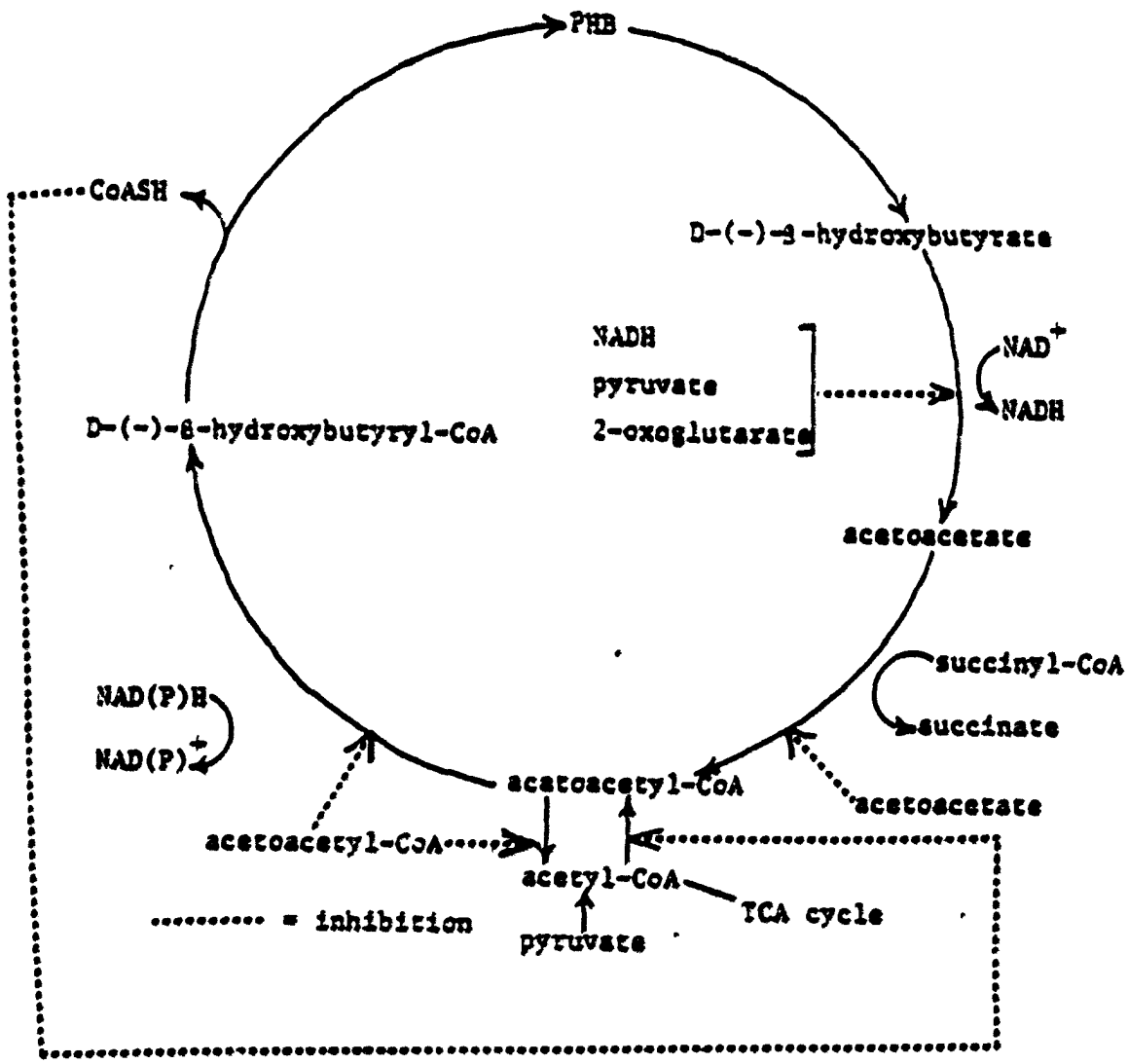


FIGURE 7. PHB catabolism and regulation in A. beijerinckii (Dawes, 1975)

Hypochlorite digestion

Williamson and Wilkinson (1958) established a technique for the digestion of Bacillus cells by alkaline sodium hypochlorite (pH 9.8) resulting in the release of intracellular lipid inclusions, including PHB granules. After digestion in the hypochlorite reagent for two hours at 37° C, the granules were dialyzed against distilled water for 24 hours (removing the hypochlorite reagent and volutin granules). $Na_2S_2O_3$ was used to remove excess chlorine. The precipitate was redialyzed, lyophilized, and extracted several times with anhydrous ether. This fraction was soluble in chloroform, and was identified as PHB.

The hypochlorite digestion method has been modified to include washings with acetone and absolute ethanol, as well as ether (Law and Slepecky, 1961).

The hypochlorite extraction method is sometimes modified by the addition of a solvent extraction for several days in a Soxhlet apparatus (Delafield et al., 1965; Smibert and Krieg, 1981). A recent modification of the hypochlorite digestion method is presented schematically in Figure 8. A further modification by Nuti et al. (1972) is also presented.

Chloroform extraction

Extraction methods based on the solubility of PHB in boiling chloroform were common in early studies of the polymer (Weibull, 1953;

Fukui et al. (1976)

10 g cells (*Zoogloea ramigera*) suspended in 100 ml of sodium hypochlorite solution (10% active chlorine) at 30° C for 18 hours.

↓

White precipitate washed 3x with 300 ml water. Dried over P_2O_5 in a desiccator and dissolved in 50 ml of chloroform.

↓

Chloroform extract filtered (sintered glass funnel) and concentrated to 5 ml in a rotatory evaporator.

↓

Extract mixed with 30 ml ethanol, and stored at -20° C overnight. Precipitate collected by centrifugation and redissolved in chloroform.

↓

Solution mixed with 30 ml ethanol per 5 ml chloroform. PHB precipitated.

↓

PHB washed with 50 ml each acetone and ether and dried over P_2O_5 in a desiccator. Purity reported as approximately 98%.

Nuti et al. (1972)

10 g cells (*Azotobacter chroococcum*) lyophilized and suspended in one liter of sodum hypochlorite (commercial bleach). Shaken 8 hours at 36° C.

↓

Digest centrifuged and washed 3x with distilled water, acetone, and absolute ethanol. Precipitate suspended in thioglycollic acid solution[1]

↓

Thioglycollic acid solution overlayed with peanut oil and incubated for 16 hours at 50 ° C.

↓

Oil removed and solution centrifuged. Precipitate lyophilized and retreated with thioglycollic acid reagent (30 ml per 1 gram of cells).

↓

Thioglycollic acid solution removed by filtration, PHB deposited on filter.

↓

PHB extracted through filter pad with small portions of hot chloroform.

↓

Polymer dried to a film at room temperature and collected for analysis.

---

[1]Thioglycollic acid reagent: EDTA 29.2 g, thioglycollic acid 4.5 ml, deionized water 1000 ml. Adjusted to pH 8.5 with 10% NaOH.

FIGURE 8. Two hypochlorite extraction methods for the isolation of PHB.

Macrae and Wilkinson, 1958). While the chloroform extraction method is considered to be more cumbersome than the hypochlorite digestion methods, it is still used occasionally, particularly where a high molecular weight polymer is desired (Alper et al., 1963; Akita et al., 1976).

The chloroform extraction method involves treatment of dried cells with hot chloroform or dichloromethane, with removal of the insoluble cell components by filtration. PHB is precipitated by mixing the filtrate with n-hexane or diethyl ether. The polymer is then collected by drying the solution, often under reduced pressure, to yield a thin film (Alper et al., 1963; Akita et al., 1976).

Alper et al. (1963) compared the chloroform extraction method and the hypochlorite digestion method and found that PHB isolated by the hypochlorite method had a molecular weight of approximately 10,000 daltons and a viscosity of 0.45 dl/g while PHB isolated by chloroform extraction had a molecular weight of 128,000 daltons and a viscosity of 5.6 dl/g. It should be noted however, that PHB isolated by the chloroform extraction method for this study was obtained from Rhizobium and its molecular weight determined by osmometry, while PHB isolated by hypochlorite digestion was obtained from Bacillus cereus and its molecular weight determined by the Archibald method. Molecular weights of up to 3.39×10^6 for Azotobacter PHB isolated by solvent extraction have been reported (Akita et al., 1976), although ranges of 60,000 to 250,000 daltons are more common (Lundgren et al., 1965).

The hypochlorite method is considered to produce polymer of smaller molecular weights (Dawes, 1975), but gives higher total yields as a percentage of cell weight (Williamson and Wilkinson, 1958).

Recently, a method of purifying PHB by density gradient ultracentrifugation has been reported. Use of this method produced high-purity (95%) PHB from Bacillus thuringiensis in NaBr gradients. The density gradient ultracentrifugation method seems to excel in the production of high-purity high-molecular weight PHB in both granular and nongranular forms. This method is apparently not suited for the isolation of PHB in large quantities (Nickerson, 1982).

Assay

Early assays of PHB depended upon the solubility of the polymer in boiling chloroform (Lemoigne, 1926), assuming that all other cell components could be removed by extraction with other solvents, or by measuring the turbidity of lipid granules after hypochlorite digestion. This latter method assumed that all other cell components would be dissolved by the hypochlorite (Williamson and Wilkinson, 1958).

Slepecky and Law (1960a) developed a method based on the dehydration of PHB in sulfuric acid to crotonic acid with determination of the concentration of the resulting crotonic acid by spectrophotometric assay at 235 nm (the absorption maximum of crotonic acid dissolved in concentrated H_2SO_4). This method was later published in greater detail (Law and Slepecky, 1961). An assay of PHB by infrared spectroscopy has

also been developed, but has not been generally accepted (Blackwood and Epp, 1957; Haynes et al., 1958).

The crotonic acid assay of Law and Slepecky was modified by Ward and Dawes (1973) to allow the assay to be performed with small samples on glass fiber disks. In the disk assay, the bacterial cells are placed directly on a glass fiber disk and all extractions are performed on the disk with small quantities of reagent. The crotonic acid prepared from the PHB on the disks was removed and assayed spectrophotometrically.

Braunegg et al., (1978) developed a method for the assay of PHB by gas chromatography of PHB extracted from whole cells. A methyl ester of 3-hydroxybutyric acid is formed by either acid or alkaline methanolysis. Stan and Scheutwinkel-Reich (1979) further developed the gas chromatographic methods for the analysis of PHB by comparing various ionization detection techniques, recommending a chemical ionization method. Gas chromatography of PHB has been optimized for untreated environmental samples (Apostolides and Potgieter, 1981), and an analysis based on gas-liquid chromatography has been reported (Findlay and White, 1982).

PHB has traditionally been detected in bacterial cells by staining the native granules with Sudan Black B (Burdon, 1946; Lemoigne et al., 1949; Smibert and Krieg, 1981). This method is of use only for native (nonextracted) granules, as purified PHB samples do not stain well with Sudan Black B (Dawes and Senior, 1973; Nickerson, 1982).

Recently, a fluorescent stain for PHB using Nile Blue Sulfate has been reported (Ostle and Holt, 1981, 1982). This stain is based on the staining of neutral lipids which are liquid at the staining temperature by the oxazone form of Nile Blue A, Nile Pink (Thompson, 1966; Lille, 1977). Nile Blue A has previously been used as a histological stain for prepared tissue sections (Smith, 1908; Cain, 1950). The Nile Blue A fluorescent stain for PHB is a more specific stain for PHB than Sudan Black B, and is less easily washed from the cells by decolorization procedures (Ostle and Holt, 1982).

Properties of PHB

PHB isolated from bacteria has a molecular weight of between 1,000 and 250,000 daltons depending upon the extraction method used and the stage of growth of the producing organism (Dawes, 1975). Occasionally, much higher molecular weights have been reported (Akita et al., 1976). The polymer has a melting point between 160° C and 172° C depending upon the chain length (Dawes, 1975).

PHB is a water-insoluble compound that is only weakly ionic (Kannan and Rehack, 1970) and exerts very little osmotic pressure (Dawes, 1975). PHB has been reported to be insoluble in ethyl-ether and petroleum ether (Schlegel et al., 1961a,b), acetone, ethanol, carbon tetrachloride, and sodium hypochlorite solutions, as well as dilute mineral acids (Williamson and Wilkinson, 1958; Dawes, 1975). The polymer is soluble in chloroform, glacial acetic acid, pyridine, octyl alcohol, 0.5 N aqueous phenol, 1 N NaOH, and triolein (Williamson and Wilkinson, 1958).

PHB is also soluble in dichloromethane (Akita et al., 1976).

Alper et al., (1963) studied the crystal structure of PHB and established that the polymer is optically active with a weak negative rotation at 589 nm. These researchers also reported the first x-ray diffraction data for PHB crystals, and concluded that PHB existed in a regular helical formation. Electron microscopy of PHB crystals showed a characteristic "lamellar" structure which was interpreted as polymer single crystals with folded chains at right angles to the lamellar surface (Alper et al., 1963).

Okamura and Marchessault (1967) and Cornibert and Marchessault (1972) determined the conformation of PHB, indicating a right-handed helix with a fiber repeat of 5.96 A. Marchessault et al. (1970) performed studies of intrinsic viscosity, sedimentation analysis, and optical rotatory dispersion and proposed that the PHB helix behaved as a rigid rod with a sharp helix/coil transition. The model proposed involved folded helical segments (Cornibert et al., 1970). Akita et al. (1976) disagreed with these results, and being unable to duplicate the results of Cornibert, Marchessault, and Okamura, proposed instead that PHB exists as a random coil in the systems used by these researchers.

Delsarte and Weill (1974a) used shift reagent methods to confirm previous x-ray diffraction and conformational energy calculations in the determination of the internal rotation angles for the PHB helix. Their calculated angles are presented in Figure 9.

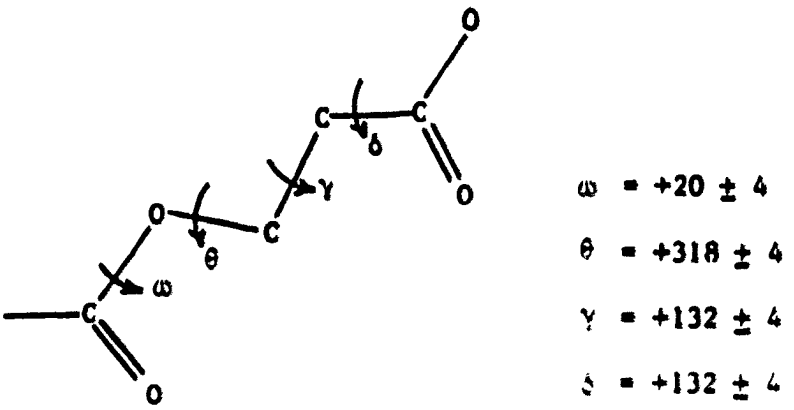


FIGURE 9. The internal rotation angles of the PHB helix (Delsarte and Weill, 1974a).

Cornibert and Marchessault (1972) proposed several models of the PHB helix based on various internal rotation angles, but none correspond exactly to the angles calculated later by Delsarte and Weill (1974a). Delsarte and Weill (1974b) also reported that PHB retained its helical conformation in chloroform and trifluoroethanol and that the optical rotatory dispersion is solvent dependent.

Coulombe et al. (1978) used high pressure liquid chromatography to study the PHB molecule and reported that the method was useful for determining the proportion of modified end units and molecular weight distributions, as well as the degree of polymerization.

Potential uses of PHB

The potential uses of PHB as an industrial product have not been well investigated, although it is known that PHB is produced in large

quantity by some organisms under laboratory conditions (Dawes, 1975). PHB isolated from various bacteria has been converted to crotonic acid, crotonic acid amine, and β-hydroxybutyrate, usually for assaying the polymer (Alper et al., 1963).

Casida (1968) speculated that PHB might be of industrial use but offered no specific suggestions as to its use. However, compounds related to PHB and its breakdown products have been used in brake fluids, gasoline additives, antibiotic recovery, and in the prevention of milk fat depression in cattle (Casida, 1968; Young, 1975; Bonner et al., 1976).

The stereospecificity of PHB and many of its degradation products makes the production of organic compounds of one steric configuration from PHB a possibility. Although these compounds can be synthesized in vitro, the need to resolve the resulting racemic mixtures makes their production expensive (G. A. Russell, Department of Chemistry, Iowa State University, Ames, personal communication, 1979). It has been suggested that PHB could be used to increase the recovery of crude oil from marginal wells, but it appears that this has not been attempted to date (Higgins and Hill, 1979; I. J. Higgins, Biological Laboratory, University of Kent at Canterbury, Kent, U. K., 1979).

The possibility of using PHB as an industrial plastic has been known for some time, especially if various plasticizers are used to improve the plastic qualities of the polymer by increasing its flexibility and elasticity. Two patents for the preparation of plastics from PHB were

granted in the early 1960s, but no further work in this field has been published (Baptist, 1962; Baptist and Werber, 1965).

The existence of PHB has been used as a taxonomic criterion for the classification of bacteria, since this characteristic appears to be stable (Haward, 1959). The ability to degrade PHB suspensions has been used in the classification of the genus Pseudomonas (Doudoroff and Palleroni, 1974; Smibert and Krieg, 1981). It must be noted that the ability to produce PHB is the only characteristic which is useful in a taxonomic sense, as the amount of PHB found in an individual cell is highly variable, depending upon the stage of growth, cultural conditions, and other variables (Stockdale et al., 1968). While these tests are found in various taxonomic schemes, they are seldom used, due to the difficulty in preparing PHB and the fact that PHB is not commercially available (Smibert and Krieg, 1981).

Artificial Synthesis

The artificial synthesis of PHB has proven difficult and was only recently accomplished (Cornibert and Marchessault, 1972; Marchessault and Faure, 1974). Agostini et al. (1971a,b) reported the production of the monomer (β-hydroxybutyrate) from which a racemic D-L PHB was synthesized.

Shelton et al. (1971) synthesized optically active D-poly-β-hydroxybutyrate by the polymerization of the optically active monomers (D-(-)-β-butyrolactone) obtained from crotonic acid. The

polymer was reportedly almost identical with biologically produced PHB and had a melting point of 168° C and a viscosity of 0.60 dl/g (as compared to a viscosity of 0.04 to 11.95 dl/g reported for biologically produced PHB). The rotatory power of the artificial polymer was somewhat less than that for natural PHB (+19 vs +44 at 300 nm). X-ray diffraction measurements of the artificial polymer were identical to those obtained with bacterial PHB (Shelton et al., 1971).

The synthetic procedure used by Sheldon et al. was an involved process due to the need to resolve the racemic mixture obtained when the monomer was produced from crotonic acid. The polymerization sequence itself was also complex, involving a triethylaluminum catalyst and a reaction period of several days at temperatures varying from room temperature to 0° C in a nitrogen atmosphere. No cost figures were given for this synthesis, but at present it would not appear to be competitive with biological processes (Shelton et al., 1971).

Biological Synthesis of PHB in Large Quantities

The majority of researchers reporting the isolation of PHB in the laboratory have utilized strains of Azotobacter or Bacillus. Azotobacter beijerinckii has been reported to accumulate PHB up to 70% of the dry weight of the cell (Dawes, 1975).

The conditions under which Azotobacter will accumulate PHB in laboratory scale culture have been investigated by Stockdale et al. (1968), Oppenheim and Marcus (1970), Nuti et al. (1972), Senior et al.

(1972), Dawes (1975), and Ward et al. (1977). The majority of work on PHB in Azotobactar spp. has been performed in the laboratory of E. A. Dawes at the University of Hull in England.

Dawes summarized the research performed in his laboratory in 1975, and reported that A. beijerinckii accumulated the greatest quantities of PHB in batch culture towards the end of the logarithmic growth phase. In chemostat studies it was determined that Azotobacter did not accumulate PHB when nitrogen was limited and that oxygen-limited cultures accumulated large amounts of PHB in an inverse relationship to the specific growth rate (Dawes, 1975). Senior et al. (1972) reported that under conditions of oxygen limitation, 74% PHB (cell dry weight) was accumulated in batch cultures and up to 50% (cell dry weight) in chemostats. The effect of oxygen limitation is believed to be due to the functioning of PHB as an electron sink (Senior et al., 1972; Senior and Dawes, 1973). These results were confirmed by Ward et al. (1977).

Oppenheim and Marcus (1970) reported that Azotobacter vinelandii grown without a nitrogen source other than atmospheric nitrogen did not accumulate detectable amounts of PHB. PHB accumulation was obtained when ammonium chloride was supplied, but nitrate or amino acids were not acceptable nitrogen sources. Stockdale et al. (1968) reported that PHB was accumulated in Azotobacter, Beijerinckia, and Derxia with atmospheric dinitrogen as the sole nitrogen source.

Nuti et al. (1972) treated Azotobacter chroococcum with phenylacetic acid and discovered that the average molecular weight of PHB isolated

from these cells was greater than that of PHB isolated from untreated cells. The phenylacetic acid treated cells were also longer (7.1 μm vs 2.7 μm), but septa formation, cell walls, membranes, and mesosomes were apparently not affected. The phenylacetic acid did not enter the PHB molecules, but apparently affected other cell pathways, and did enter cell wall constituents and cytoplasmic proteins.

PHB production in Bacillus cereus and Bacillus megaterium was studied by Williamson and Wilkinson (1958). These researchers avoided the problem of PHB catabolism during sporulation by employing an asporogenous mutant of B. cereus. This method was also employed by Emeruwa and Hawirko (1973) with an asporogenous Clostridium botulinum strain. Williamson and Wilkinson (1958) reported that a Bacillus cereus strain accumulated up to 42.6% PHB (dry weight) while PHB contents of up to 60% for B. cereus were reported by Alper et al. (1963). Thus, strains of Bacillus would appear to be capable of producing PHB in large quantities.

Sakharova (1977) performed chemostat studies with Bacillus megaterium in a citrate-limited synthetic medium. PHB was accumulated in increased amounts at alkaline pH values (7.6 to 9.6 depending upon growth rate), and it was found that low growth rates favored PHB accumulation.

Bacillus megaterium has not been used to produce PHB in large quantities, but its usefulness as a source of protein has been investigated. Zalabak et al. (1975) determined that B. megaterium could be produced in quantities of 15-20 g dry weight per liter of minimal

medium with cornsteep liquor. These cells contained 8-9% nitrogen, 40% protein, 30% PHB, and 10-14% RNA. The possibility of producing feed protein and PHB via the same fermentation is attractive, but was not mentioned by these authors.

Although Azotobacter and Bacillus spp. are the most frequently studied PHB producers, PHB extraction procedures for an antibiotic producing strain of Streptomyces have been established on a laboratory scale (Kannan and Rehack, 1970). It is appealing to contemplate the coproduction of streptomycin and related antibiotics with PHB, but it appears that the organisms utilize PHB for antibiotic production, with PHB content inversely proportional to antibiotic production levels (Kannan and Rehack, 1970).

If the presence of PHB in Saccharomyces cerevisiae can be confirmed (Nuti and Lepidi, 1974), this organism might well prove to be an attractive industrial source of PHB. Industrial fermentations for Saccharomyces are well-established, and it might be possible to co-produce PHB, high quality protein, or yeast extract products.

Few other PHB-containing organisms have been produced on an industrial scale, although Clostridium spp. were used in large-scale fermentations to produce acetone and butanol during the First World War (Casida, 1968). Some Bacillus spp. have also been produced on an industrial scale for their enzymes and for use as an insecticide (Casida, 1968). The production of Hydrogenomonas on a pilot-plant scale has also been reported (Foster and Litchfield, 1964). A patent exists

for the preparation of PHB from Bacillus megaterium and Rhodospirillum rubrum although the choice of the latter organism for a large-scale fermentation is puzzling (Baptist, 1962). Some members of the genus Pseudomonas have been grown in large-scale fermentations for the production of their extracellular polysaccharides, but it is not known if these strains produce PHB (J. S. Racciato, Kelco corp., San Diego, California, personal communication, 1982).

Encystment

The genus Azotobacter is known to form cysts which function as dormant survival structures. This process has been reviewed by Sadoff (1975) who concentrated on A. vinelandii. The process of encystment involves a definite life cycle which includes both motile vegetative stages and encysted stages. The encysted cells have a definite intine and exine which can be observed microscopically. Encystment occurs at the end of log growth and involves the transformation of the generally rod-shaped vegetative form to a spheroid encysted form containing novel lipids and carbohydrates. Motility is lost upon encystment. PHB is present in both encysted and pre-cyst vegetative forms (Pochon et al., 1948, Sadoff et al., 1971).

In order for encystment to occur, Sadoff (1975) has stated that Azotobacter cells must be grown in a nitrogen-free, carbohydrate-limited environment. When cells begin encystment, they lose the ability to fix atmospheric dinitrogen. However, when nitrogen is replaced with argon, no cell growth or encystment occurs, even if encystment has already

begun prior to the removal of nitrogen (Hitchins and Sadoff, 1973; Sadoff, 1975). Encystment may be induced chemically by the introduction of n-butanol, β-hydroxybutyrate, or crotonate to cells washed of all carbohydrate from the growth medium. These inducers appear to be chemical precursors of PHB (Sadoff, 1975).

MATERIALS AND METHODS

Bacterial Cultures

Thirty-three strains of Azotobacter chroococcum, four strains of Azotobacter beijerinckii, and three strains of Azotobacter vinelandii were employed in this project. All Azotobacter spp. were isolated from Central Iowa or Central Florida soils. These cultures were isolated by a modification of well-known methods (Aaronson, 1970; Krieg, 1981; Durand et al., 1982). Soil, (approximately 1 gram), was inoculated into 100 ml of Nitrogen-Free Ethanol Broth (NFE) in 250-ml screw-cap prescription bottles, (Table 2) and incubated for twelve days at room temperature. Cultures were isolated by streaking the incubated broth on nitrogen-free solid medium (Mod I) (Table 3) and incubating for four days at 30° C. Isolated colonies were checked for typical Azotobacter morphology by phase-contrast microscopy as well as for the presence of multiple, highly refractile granules characteristic of PHB.

These presumptive azotobacters were tested for the ability to produce PHB on nitrogen-free media by staining with Sudan Black B or Nile Blue Sulfate. Cultures were rated + to ++++ by the presence of stained granules (presumably PHB) under microscopic observation.

Cultures were transferred to three sets of nitrogen-free agar slants (Mod I, later Mod IV) in screw-cap culture tubes. One N-free agar slant was covered with sterile mineral oil and maintained as a stock culture at room temperature. The second slant was maintained at 30° C as a

TABLE 2. Nitrogen-Free Ethanol Broth

Ethanol[a,b]	4.0 ml
$MgSO_4 \cdot 7H_2O$	0.2 grams
K_2HPO_4	1.0 g
$FeCl_3 \cdot 6H_2O$	0.005 g
$MnSO_4 \cdot 4H_2O$	0.001 g
$CaCO_3$	1.0 g
$NaMoO_4$	0.001 g
Deionized water[c]	1000 ml

[a] $MgSO_4$, $FeCl_3$, $MnSO_4$ from Fisher Scientific. K_2HPO_4, $CaCO_3$, $NaMoO_4$ from J. T. Baker Chemical Co.

[b] Ethanol from Aaper Alcohol and Chemical Co. Added after autoclaving and cooling.

[c] Final pH = 7.4

working culture. The third slant was covered with dimethylsulfoxide (5% aqueous) (DMSO, Fisher) in screw-cap culture tubes and stored at -76° C in an ultracold freezer (Kelvinator Ultracold, series 100, Kelvinator Corp., Manitowoc, WI). DMSO was sterilized by filtration through a 0.02 Selas filter (Selas Flowtronics, Spring House, PA). Working cultures were recultured from stock cultures at one-month intervals. Stock cultures maintained under mineral oil were recultured at six-month intervals. Stock cultures in DMSO were never recultured (Lapage et al., 1970).

Isolates producing large amounts of PHB were characterized to the species level (where possible) using the biochemical tests suggested by Johnstone (1974) and Thompson and Skerman (1979). Cultures were designated Az1-Az40. Az indicates the culture is a member of the genus Azotobacter and the number refers to the order in which the organism was

TABLE 3. Nitrogen-Free Media[a]

	Mod I	Mod II	Mod III	Mod IV	Mod V[e]
Glucose[b]	10 g	10 g	25 g	23 g	23 g
K_2HPO_4	0.5	0.5	1.0	1.0	1.0
$MgSO_4 \cdot 7H_2O$	0.2	0.2	0.2	0.2	0.2
NaCl	0.2	0.2	0.1	0.1	0.1
$FeCl_3 \cdot 6H_2O$	0.005	0.005	0.005	0.005	0.005
$MnSO_4 \cdot 4H_2O$	0.001	0.001	0.001	0.001	0.001
$CaCO_3$	10.0	1.0	0.5	0.5	0.5
$NaMoO_4 \cdot 2H_2O$	---	---	1.0 mg	1.0 mg	1.0 mg
Riboflavin	---	---	---	1.0 mg	1.0 mg
Biotin	---	---	---	1.0 mg	1.0 mg
Pantothenic acid[d]	---	---	---	1.0 mg	1.0 mg
Vitamin solution[d]	---	---	3 drops	---	---
KNO_3	---	---	---	---	1.0 g
Deionized water[c]	to 1000 ml				

[a]For solidified media, 1.5% Bacto Agar (Difco) added.

[b]Autoclaved separately.

[c]Final pH = 7.4

[d]Thiamine HCl 10 mg, pyridoxal HCl 0.8 mg, ribioflavin 10 mg, biotin 1.0 mg, nicotinic acid 15 mg, folic acid 0.5 mg: per 100 ml.

[e]Glucose, $MgSO_4$, NaCl, $FeCl_3$, $MnSO_4$ from Fisher Scientific. K_2HPO_4, $CaCO_3$, $NaMoO_4$ from Baker. Vitamins from Sigma Chemical Co.

isolated.

Bacillus megaterium KM, an asporogenous mutant, was obtained from I. K. Nakamura, U. S. Department of Agriculture, Northern Regional Research Center, Peoria IL. The strain designation of the B. megaterium KM culture was NRRL B-3694 (hereafter called B. megaterium KM). This strain was originally obtained from J. T. Wachsmann, of the University of Illinois in 1969. B. megaterium KM was obtained as a lyophile and was rehydrated and maintained in Trypticase Soy Broth (BBL, Cockeysville, MD) supplemented with 0.2% yeast extract (Difco, Detriot,

MI) (TSYE). Stock cultures of B. megaterium KM were maintained on TSYE slants in screw-cap culture tubes under sterile mineral oil at room temperature, and at -76° C on TSYE slants in screw-cap culture tubes with DMSO (as for azotobacters).

Bacillus cereus, Dermatophilus, and Pseudomonas strains were isolated from Central Iowa soil and were grown and maintained on TSYE. Stock cultures were maintained by the same methods used for Bacillus megaterium KM with the exception of Dermatophilus which was maintained on TSYE and transferred biweekly. Psuedomonas was isolated on Grant's medium (Grant and Holt, 1977) and was identified to the genus level by the methods recommended by Doudoroff and Palleroni (1974). Bacillus cereus and Dermatophilus were isolated on TSYE and identified according to the methods recommended by Gibson and Gordon (1974) and Gordon (1974).

Bacillus, Pseudomonas, and Dermatophilus were also tested for PHB production on TSYE slants at 35° C by staining the cultures with Sudan Black B or Nile Blue Sulfate (as for azotobacters).

Assay of PHB

Staining

PHB granules in cells were stained by Sudan Black B (Harleco, Philadelphia, PA). Sudan Black B was used as a 0.3% solution in ethylene glycol (Eastman, Rochester, NY). The stain was performed

according to the method of Burdon (1946). This involves staining a heat-fixed smear with Sudan Black B solution for fifteen minutes, decolorizing the stained smear with xylol (Baker), and counterstaining with 0.5% aqueous Safranin (Matheson, Coleman and Bell, Norwood, OH) for one minute. The stain is observed under a high-power oil immersion lens by ordinary light microscopy.

PHB was also stained with Nile Blue Sulfate (NBS) (Matheson, Coleman and Bell). NBS was prepared as a 1% aqueous solution and filtered prior to use (Whatman No. 1 paper). Mild heating was occasionally necessary to fully dissolve the stain. Acid reflux to produce the oxazone form of NBS was not required.

Staining with NBS was performed by immersing heat-fixed bacterial smears on glass microscope slides in Coplin staining jars filled with 1% NBS, for ten minutes at 55°C. The stained slides were washed with tap water to remove excess stain and decolorized in 8% aqueous acetic acid (Fisher) for one minute. The decolorized smear was washed briefly in tap water and blotted dry with bibulous paper. The stain was re-moistened with deionized water, covered with a No. 1 glass coverslip, and observed with a Nikon Labphot microscope with an episcopic fluorescence attachment. The Nikon blue excitation method, which provides an excitation wavelength of approximately 460 nm, was used. PHB granules fluoresce as a bright orange with this method.

PHB was removed from the cell by extracting heat-fixed smears on glass microscope slides overnight with chloroform (Fisher) or

dichloromethane (Mallinckrodt, St. Louis, MO) and the extracted cells were stained with NBS. Glycogen powder (oyster and fungal) (BBL, Baker) and metachromatic granules in Corynebacterium diphtheriae were also stained in order to establish whether NBS stained other common cell inclusion bodies in the same manner as PHB. Heat-fixed smears on glass microscope slides were soaked overnight in benzene or ether (Fisher) to determine if the stained inclusions were affected by these solvents (Ostle and Holt, 1981; 1982).

Heat-fixed smears on glass microscope slides of PHB-containing organisms were also stained with the dyes listed in Table 4 to determine if these dyes stained PHB granules. The methods used were those established for the use of each dye (Thompson, 1966; Lille, 1956, 1977; McManus and Mowry, 1960).

Rapid replica assay

Soil isolates of Azotobacter spp. were assayed for the presence of PHB by transferring the colonies from the medium on which they were originally isolated to Nitrogen-Free Mod I Agar (NFA) using sterile wooden toothpicks. Toothpicks were sterilized by autoclaving for 30 minutes. The colonies were transferred in a grid pattern with the colonies approximately 2 cm apart. This master plate was incubated at 30° C for 48 hours.

After the colonies were approximately 5 mm in diameter, the entire plate was replicated onto a sterile 9.0 cm glass-fiber filter disk

TABLE 4. Dyes Examined for the Ability to Stain PHB

Acridine Orange (K&K Labs)
Basic Fuchsin (Eastman)
Janus Green (National Aniline & Chemical Co.)
Luxol Fast Blue (Hopkins & Williams)
Meldola Blue (Hopkins and Williams)
Methylene Blue (Eastman)
Neutral Red (Harleco)
Nile Blue A (Matheson, Coleman and Bell)
Oil Red O (Hopkins & Williams)
Osmium tetroxide (Sigma)
Phosphine GN (Allied Chemical)
Rhodamine B (Eastman)
Rhodamine Red (Eastman)
Rose Bengal (Matheson, Coleman and Bell)
Titan Yellow (Eastman)

(Whatman GF/A) by attaching the filter to a wooden plate-replicating template with double-stick tape and pressing the disc onto the master plate.

The glass-fiber filter (now impregnated with replicas of the colonies) was immersed in a glass petri dish filled with 1% aqueous NBS, and stained at 55° C for fifteen minutes. The stained filter disk was washed gently in running tap water for one minute, decolorized in 8%

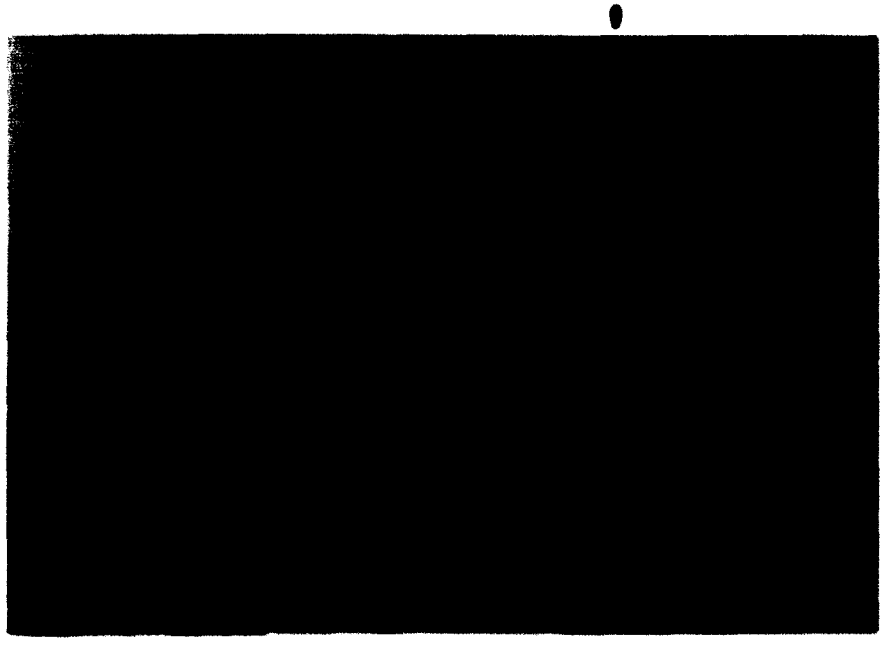
aqueous acetic acid for ten minutes, and washed in flowing tap water again for one minute. The moist disk was then observed under near-ultraviolet light in the apparatus shown in Figure 10. Colonies containing PHB fluoresced orange.

The original colonies (on NFA) corresponding to fluorescing colonies were examined with phase-contrast microscopy and by staining heat-fixed smears on glass microscope slides with NBS to confirm the presence of PHB granules. Colonies containing PHB granules were retained for further study on NFA slants.

Qualitative assay

Strains producing PHB were grown in 100 ml of appropriate media (nitrogen-free media at 30° C for azotobacters, TSYE at 35° C for Bacillus, Dermatophillus, and Pseudomonas spp.) in 250-ml Erlenmeyer flasks with shaking. All shaking in this study was performed on a Cutler-Hammer shaker at 120 rpm. The flasks were stoppered with gauze filter pads held in place by wire clips to obtain good aeration.

PHB was assayed by a modification of the method of Law and Slepecky (1961). One-milliliter samples (more in sparse cultures) of bacterial cultures were centrifuged to pellet the cells, washed in 8% aqueous acetic acid, recentrifuged, and resuspended in 9.0 ml of 5.25% sodium hypochlorite (Fisher, or Des Moines commercial bleach). The acid wash was performed to remove excess calcium carbonate present from the growth medium. This mixture was incubated at room temperature for 24 hours.



A. Specimen chamber (opening in side to insert plate)

B. Ultraviolet barrier filters (Leitz K530, Leitz, FRG)

C. Near UV light source (Leitz, model 250)

D. Power Source (Electro Powerpacs Corp., Cambridge MA)

FIGURE 10. Replica Viewing Device

After incubation the mixture should contain a white precipitate. The mixture was centrifuged in glass centrifuge tubes (Pyrex graduated conical, Corning Glass Works, Corning, NY) at high speed in a table-top centrifuge (Sorvall angle-head) for 30 minutes. The supernatant was discarded and the pellet was washed three times in distilled water, two times in 95% ethanol, two times in acetone, and two times in a 1:1 mixture of acetone:ether.

The final pellet was dried overnight in a vacuum or for three hours in a vacuum oven (Arthur H. Thomas Co., Philadelphia,PA) at 90° C. The precipitate, a white powder, was dissolved in 10 ml of concentrated reagent-grade sulfuric acid (Fisher), and this mixture was heated in a steam bath until the mixture boiled for at least ten minutes. This treatment reduces the polymer to crotonic acid. The sulfuric acid/crotonic acid mixture was cooled, transferred to a square quarts cuvette (Beckman, Palo Alto, CA), and the optical density of the mixture was determined at 235 nm against a concentrated sulfuric acid blank in a Beckman DB double beam spectrophotometer. The absorbance reading thus obtained was compared to a standard curve obtained by the identical assay method as described above, substituting DL-β-hydroxybutyrate (Matheson, Coleman and Bell) in 0.05-1.0 g quantities (in 0.05-g increments) for the PHB.

An indication of the purity of the preparation may be obtained by using the spectrophotometer in a scanning mode, and obtaining the UV spectrum (from 200-280 nm) of the preparation. A broad peak at 235 nm

characteristic of crotonic acid should be observed.

The amount of polymer isolated may be determined by weighing the precipitate prior to degradation in sulfuric acid. The wet weight of the cells may easily be obtained by weighing pre-weighed centrifuge tubes containing the packed cells before treating the cells with hypochlorite. PHB was reported as percent cell wet weight or percent cell dry weight (for the latter determination, a second 1.0-ml sample was obtained, washed as above in 8% aqueous acetic acid, washed three times in PBS (pH 7.2), dried overnight in a vacuum oven at 100° C, and weighed to determine cell dry weight).

Fluorometric assay

A fluorometric assay based on the selective fluorescent staining of PHB granules by NBS was performed with Azotobacter. Azotobacter cultures in Mod III medium were grown for 72 hours at 30° C with shaking in 500-ml Erlenmeyer flasks closed with gauze pads held in place by wire clips. 1.0-ml samples were removed before inoculation and every two hours from eight hours to the end of the 72-hour growth period. These samples were placed in one-dram screw-cap glass specimen vials (Kimble). One drop of 37% formaldehyde (Fisher) was added to each vial to stop growth, and the vials were tightly capped and stored at 4° C until analysis could be performed.

When all samples had been collected, 0.1 ml of 0.01% NBS was added to each tube, and after fifteen minutes at room temperature fluorescence at

430 nm was measured with an excitation wavelength of 362 nm on a fluorimeter constructed by J. G. Foss, Department of Biochemistry and Biophysics, ISU.

The fluorometer was operated in a scanning mode to determine the optimum excitation wavelength and the wavelength of the fluorescence peaks. Detected fluorescence was reported as generated current in amperes at the detector. NBS levels of 0.001, 0.01, 0.1, and 1.0% were used to determine the optimum concentration for PHB staining.

Samples were taken from several specimen vials and examined with a Nikon epifluorescence microscope to determine whether visible granules within the cells were fluorescing.

Chromatography

Paper chromatography of isolated, purified PHB was performed by placing three drops of 1% PHB (w/v) dissolved in chloroform on Whatman chromatography paper (Whatman 3MM) and allowing the spot to air dry. Ascending chromatography was performed by placing the paper in a sealed glass jar (four quart size), with an appropriate solvent mixture. Chloroform (Fisher), dichloromethane (Mallinckrodt), ethylene carbonate (Fisher), propylene carbonate (Fisher), chloroform:dichloromethane (1:1), dichloromethane:benzene (Fisher) (1:1), and ether (Fisher):chloroform were used as solvents for chromatography. Chromatography was performed at 55° C or room temperature until the solvent front reached the top of the paper. The chromatographs were

air-dried, stained with 1% NBS, decolorized with 8% aqueous acetic acid as described for the replica-staining technique, and observed under the near-UV viewing device previously described. Chromatography was also performed using glass-fiber filter paper (Whatman) in smaller glass jars (one quart size).

Sudan Black B was used to stain some chromatographs because of its ability to stain PHB. Chromatographs were stained with 0.5% Sudan Black B and decolorized in xylol for one hour.

Isolation of PHB from Cells

Hypochlorite digestion

The isolation of PHB by hypochlorite digestion was performed by a modification of the method of Law and Slepecky (1961). This modified extraction method is presented schematically in Table 5.

This extraction method was chosen because of its ability to isolate and recover large quantities of PHB.

The hypochlorite:cell ratio of the scheme in Table 5 was varied between 15:1 and 3:1 in the following ratios; 15:1, 12:1, 10:1, 6:1, 4:1, 3:1 in an effort to determine the optimum hypochlorite concentration. Cells were isolated by centrifugation, washed once in 3% aqueous acetic acid, and recentrifuged. The initial water washes (step 1) were increased to five and decreased to zero, and the washes after treatment with hypochlorite were performed 5, 10, and 15 times in order

TABLE 5. Isolation of PHB by hypochlorite digestion

1. Collect cells by centrifugation, wash once in 8% (v/v) aqueous acetic acid, twice in nonsterile tap water.
2. Place cells in hypochlorite reagent (8:1 hypochlorite:cells)[a]. Digest 12 hours at room temperature with stirring.
3. Wash five times with tap water.
4. Wash twice each in 95% ethanol and acetone.
5. Wash once in acetone:ether (1:1).
6. Soak in acetone:ether (1:1) overnight.
7. Extract PHB in hot 1% ethylene chloride in chloroform[c].
8. Separate the viscous mixture by centrifugation or in a separatory funnel.
9. Precipitate PHB from the chloroform by mixing 2:1 chloroform-PHB: 95% ethanol or by drying overnight in a vacuum desiccator.
10. Assay precipitated PHB

[a]Hypochlorite solution is commercial bleach.

[b]The solution is stirred in an Erlenmeyer flask on a magnetic stirrer at the highest speed possible with the liquid volume/stirrer combination used.

[c]Extraction is best performed in a separatory funnel.

to determine the optimum amount of water necessary to remove hypochlorite and water-soluble cell components. The wet weight of the cells was determined after the intial water washes. Dry weight determination was also performed at this time. Samples for which dry weight was determined were washed as above, dried overnight at 50° C (or longer if not completely dry), and weighed.

The solvent washes were also varied (according to Table 24), to determine the optimum amount and type of solvents necessary to produce purified PHB. The unmodified extraction scheme (Table 5) was taken as 100%, and the amount of PHB obtained by varying the water wash or solvent wash steps was reported as a percentage of that obtained by the unmodified procedure.

In order to determine the optimum solvent mixture for the final extraction of PHB, chloroform (Fisher), dichloromethane (Mallinckrodt), propylene carbonate (Fisher), and ethylene carbonate (Fisher) were used to extract the final polymer after solvent washings. All extractions were performed with both solvents heated to boiling and solvents at room temperature. Solvents were boiled in a steam bath.

Cells were also extracted in 1N NaOH 10:1 (NaOH:cells) rather than in hypochlorite to determine whether NaOH could be used to digest the cells and release PHB granules in place of hypochlorite.

Cells for the determination of the hypochlorite extraction optima were Az3 (Azotobacter chroococcum) grown in Mod III medium at 30° C in

Fernbach flasks with shaking. The Fernbachs were stoppered with milk filters (6.5 inch disks, Kendall, Walpole, MA) held in place by rubber bands to insure good aeration. Three liters of 36-hour old cells were pooled and used as a standard cell suspension to compare the varied hypochlorite extraction schemes with the basic scheme.

In all cases, results were reported as a percentage (by weight) of the polymer isolated by the unmodified hypochlorite extraction system. The hypochlorite:cell mixtures were visually examined for the presence of color in the precipitate after incubation of the mixture. Samples of polymer from all variations of the basic scheme were assayed by degradation to crotonic acid to confirm the presence of PHB. Scanning UV spectra were obtained for the PHB:sulfuric acid degradation products to determine if absorbance peaks other than the crotonic acid peak were present.

In addition, the hypochlorite extraction method of Nuti (1972) was performed according to the instructions given by that author. Az3 cells were grown in the same manner as outlined for the standard hypochlorite extraction procedure above.

Growth Curves

Preliminary growth curves

Preliminary growth curves were performed with one liter of medium in Fernbach flasks stoppered with milk filters to insure good aeration and

shaken at 30° C. Mod I medium was used in preliminary growth curves. A 0.1% 30-hour old inoculum from a medium identical to that in which the growth curve was to be performed was used.

Samples were taken from the growth flasks at one hour intervals (one-half hour intervals in logarithmic growth phase). The optical density of each sample was determined at 660 nm in a Spectronic 20 spectrophotometer (Bausch and Lomb) with the instrument zeroed against a blank of sterilized medium. Total cell counts were obtained by microscopic observation of cell suspensions in a Petroff-Hausser counting chamber (Arthur H. Thomas Co., Philadelphia, PA) using a phase-contrast microscope. During counting, cell morphology and the presence of motile cells was noted. The presence of cysts in Azotobacter was also noted as was the production of a water-soluble brown pigment. Total viable cell counts were obtained by plating samples on Mod I agar (later Mod IV) for Azotobacter. Samples were diluted in sterile phosphate buffered saline blanks (pH 7.2), and spread plated in 0.1 ml quantities on Mod I or Mod IV agar with sterile glass spreaders.

Growth curves in fermentors

Growth curves in fermentors were performed in ten-liter New Brunswick fermentors (New Brunswick Corp., New Brunswick, NJ), and in a Bellco glass spin flask fermentor (Bellco, Vineland, NJ) with five liters of medium. Aeration in the New Brunswick fermentors was controlled by the aeration manifolds in the fermentor assembly. The temperature and

impeller rpm were also controlled by built-in features of the fermentor assembly. Air was sterilized by passage through an in-line cotton-plug filter and moistened by passage through an Erlenmeyer flask containing sterile deionized water.

In the Bellco spin flask fermentor, temperature was controlled by placing the fermentor in a 30° C walk-in incubator. Impeller rpm was controlled by using a magnetic stirrer motor (Fisher) insulated from the flask by several layers of paper. The highest possible rpm with the flask/motor combination was used. Aeration was controlled by the use of an oxygen flow meter from a veterinary anesthesia machine (Ohio Chemical & Surgical Equipment Co., Madison, WI). Air was prefiltered from laboratory lines through glass wool, sterilized by passage through a 0.45 μm membrane filter (Millipore HA, Bedford MA, or Gelman GN 6, Ann Arbor, MI) in a Gelman in-line Delrin filter holder, and humidified by passage through an Erlenmeyer flask containing sterile deionized water.

Foaming was controlled by the use of Foamkil silicon antifoam spray (National Biochemicals Corp., Cleveland, OH) or by the use of silicon antifoam A emulsion (Sigma).

Temperature was monitored by standard mercury thermometers and by a TM Sentry Q digital thermometer (Hampshire Controls Corp., Exeter, NH) with a strip-chart recorder (Canalco WT85 model SA, Canalco, Rockville, MD) modified to interface with the thermometer.

Samples were taken at one hour and one-half hour intervals as for the

preliminary growth curves except that ten milliliter samples were taken. Samples were assayed for optical density, total cells, viable cells, morphology, pigment, spores, and cysts as described for the preliminary growth curves. One-hundred-milliliter samples were obtained for PHB weight determinations at 2-hour intervals from 0-12 hours post-inoculation. Samples were also assayed for PHB as percent cell dry weight. Samples were also stained with NBS and PHB granules were observed microscopically.

Carbohydrate Sources

Screening

Cultures were screened for the ability to use various carbohydrates as sole carbon sources. The screening was performed by diluting 36-hour old bacterial cells in sterile saline blanks to cell densities of approximately 10^4 cfu/ml, and plating 0.1 ml aliquots on Mod I agar without carbohydrate. Plates were inoculated by the spread-plate technique using sterile glass spreaders, and dried at 30° C for one hour prior to the addition of carbohydrate discs. Carbohydrates in 1% aqueous solution were neutralized with sodium hydroxide or hydrochloric acid, sterilized by filtration (Millipore 0.22 μ HA filters), and placed on sterile 2 cm filter paper disks (Whatman #1). The saturated disks were placed on the inoculated Mod I medium.

The inoculated medium with carbohydrate disks was incubated at 30° C for 72 hours. A zone of growth around a disk was taken as evidence of

the ability of the tested organism to use the carbohydrate on that disk as a sole carbon source. The carbohydrates tested in this manner are listed in Table 6.

Amylase production

The ability of the isolated cultures to use starch as a sole carbon source was tested by inoculating Nutrient Agar (Difco) or Mod I agar with 0.5% soluble starch (Fisher; or Argo cornstarch, CPC International, Englewood Cliffs, NJ) replacing the glucose, with the tested strain. Where cornstarch was used, it was converted to Lintner soluble starch by mixing the starch with 1N HCl for four weeks at room temperature (Degering, 1943). The treated starch was washed until the pH increased to near-neutral. The mixture was then neutralized with NaOH. The neutralized starch was then air-dried and ground to a fine powder before addition to the basal medium.

Starch plates were inoculated with the test cultures by streaking a single 4-5 cm long line on the plate using the desired strain, and allowing the plate to incubate for 48 to 72 hours (depending upon the speed of growth). The incubated plates were placed over iodine crystals to visualize the starch, and clear areas around the colonies were taken as evidence of amylase production and starch hydrolysis (Durand et al., 1982). Cells from amylase-positive colonies were stained with NBS and examined microscopically for the presence of PHB granules.

TABLE 6. Carbohydrates Tested as Sole Carbon Sources

Arabinose (Matheson, Coleman, & Bell)
Cellobiose (Sigma)
Glucose (Fisher)
Dextrin (Pfanstiehl)
Fructose (General Biochemicals)
Fucose (Sigma)
Galactose (Pfanstiehl)
Inositol (Sigma)
Inulin (Sigma)
Lactose (Matheson, Coleman, & Bell)
Maltose (Fisher)
Mannitol (Pfanstiehl)
Mannose (Matheson, Coleman, & Bell)
Melibiose (Matheson, Coleman, and Bell)
Melezitose (Sigma)
Rhamnose (Sigma)
Ribose (Sigma)
Sorbitol (Pfanstiehl)
Sorbose (General Biochemicals)
Trehalose (Matheson, Coleman, & Bell)
Xylose (Sigma)

Xylose and cellulose utilization

Xylose was included in Mod I, Mod II, and Mod III media in 1.0% concentration, in place of other carbohydrates, to select for nitrogen-fixing organisms capable of utilizing xylose as a sole carbon source. Xylose in Mod I broth without other carbohydrates was inoculated with soil samples as described for Azotobacter isolation in NFE broth in order to isolate Azotobacter strains capable of using xylose as a sole carbon source.

The ability to use cellulose as a sole carbon source was tested by the addition of 1% (w/v) cellulose-azure (Sigma) to Mod I agar without other carbohydrates. Colonies with zones of clearing around themselves were considered to be cellulose degraders.

Production of PHB with various carbon sources

Various carbohydrates (glucose, sucrose, fructose, soluble corn starch, ethanol, inositol, mannitol, maltose, benzoate, galactose, trehalose, and rhamnose) were added in 1% (w/v) concentration to Mod III medium in place of glucose. One-liter quantites of these media were inoculated with the test strains (0.1% inoculum) and incubated with shaking at 30° C in Fernbach flasks. Cells were harvested after 36 hours of growth and cell wet weight, cell dry weight, and PHB content as percent cell dry weight was determined for each carbohydrate source.

Growth curves were performed with 1% (w/v) glucose, fructose, sucrose, and soluble corn starch in Mod III medium in place of glucose. A

tenth-percent inoculum of Az3 cells was used and the inoculated medium was incubated in one liter quantities in Fernbach flasks at 30° C with shaking. Growth was measured as optical density, total cell count, and PHB as percent cell dry weight.

Glucose was used as a carbohydrate source with its concentration varied between 0.5 and 3.4% (w/v), at levels of 0.5, 1.0, 1.5, 1.7, 1.9, 2.0, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 3.0, and 3.4% (w/v), in Mod III medium in place of the normal glucose concentration.. Each medium containing various concentrations of glucose was incubated in Fernbach flasks stoppered with milk filters at 33° C with shaking. After 36 and 60 hours of growth, the cells were collected by centrifugation (Sorvall RC5 centrifuge, GSA rotor, metal centrifuge bottles) and the total PHB content of the cells (as percent dry weight), as well as the total wet and dry weight of the cells was determined.

To determine if xylose could be metabolized by Az3 if glucose was present, 1% xylose and 0.1% glucose were used as the carbohydrate source in Mod III medium. Mod III medium with only 0.1% glucose was used as a control. Both media were placed in 100-ml quantities in 250-ml Erlenmeyer flasks stoppered with gauze pads and inoculated with log-phase glucose-grown Az3 cells at a 1% level. The cultures were incubated at 30° C with shaking for 96 hours. After incubation, the cells were harvested by centrifugation and the total cell mass as cell wet weight was determined. PHB was assayed by NBS staining.

One-half percent sucrose and 0.5% fructose was added to Mod III medium in addition to the 2.3% glucose normally found in that medium. A 1.0% inoculum of 30-hour old Az3 cells (grown in Mod III) was used. The cultures were grown in 200 ml of supplemented Mod III in 500-ml Erlenmeyer flasks at 30° C with shaking. The cells were collected by centrifugation and cell mass was determined. The cell mass obtained from supplemented Mod III was compared to the cell mass obtained from unsupplemented Mod III medium, to determine whether the additional carbohydrates resulted in increased cell mass.

Vitamin Supplements

Vitamins were screened for the ability to increase yields of cell mass and PHB in Az3 by adding neutralized, filter-sterilized vitamins in 1.0 mg/liter solutions in deionized water or 95% ethanol to sterile filter paper disks (Whatman) and placing these disks on Mod III plates inoculated with log-phase Az3. The Mod III medium was solidified with 1.5% Noble Agar (Difco), and was prepared with high-grade deionized water. The medium was inoculated by swabbing the plates for confluent growth using sterile glass spreaders. The inoculum was prepared as described for antibiotic susceptibility testing (below). Vitamin-saturated disks were placed on the inoculated plates and the plates were incubated at 30° C for 36 hours. After incubation, the plates were examined for zones of increased growth around the vitamin disks. Vitamins showing an ability to increase growth were selected for further studies in liquid media. The vitamins screened in this study are listed

in Table 7.

TABLE 7. Vitamins Screened for Effect on Growth and PHB

Thiamine (B_1) (Sigma)
Biotin (Sigma)
Nicotinic Acid (Sigma)
Riboflavin (B_2) (Sigma)
Pyridoxal (B_6) (Sigma)
Pantothenic Acid (Sigma)
Lipoic Acid (Sigma)
Tetrahyrofolic Acid (Sigma)
p-Aminobenzoic Acid (Sigma)
Cyanocobalamin (Sigma)
Folic Acid (Nutritional Biochemicals Corp.)
Molasses (R. J. Reynolds Foods Inc.)

Vitamins showing an ability to increase the growth of Az3 in the disk assay were added to Mod III medium (from which the normally present vitamins were excluded) in quantities ranging from 1,0 mg/liter to 2.5 mg/liter in 0.5 mg/liter increments. Molasses was added in 0.01% quantities to separate Mod III medium. Ashed molasses was also added in 1 ml/liter quantities (measured prior to ashing). Molasses was ashed in a crucible over a Bunsen burner. These media were inoculated with a 1% log-phase Az3 culture grown in Mod III without vitamins, and incubated with

shaking at 30° C for 36 hours. All media were used in 100-ml quantities in 250-ml Erlenmeyer flasks stoppered with gauze pads. After incubation, cells were harvested by centrifugation and cell wet weight and PHB content were determined.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed by the standard FDA disk-diffusion method on Mueller-Hinton medium (Wick et al., 1974). Only azotobacters were tested for antibiotic susceptibility. All test media were inoculated with 36-hour cultures of the strains to be tested and incubated at 35° C for 12 hours. All inocula were standardized against 0.5 ml of 1.17% barium chloride in 99.5 ml of 0.36 N sulfuric acid. The measured zones of clearing around antibiotic disks were compared to standard values (Wick et al., 1974).

Ultraviolet Mutation Procedures

Mutation of Az3 was performed by exposing cells grown for 36 hours in Mod V medium at 35° C with shaking to ultraviolet light (50 uW/cm^2) for 5, 10, and 15 seconds. Longer exposure times are needed if encysted cells are used. Exposure times were chosen on the basis of their ability to effect a six-log (or higher) kill of the exposed cells. Cells were placed in open, sterile plastic petri dishes in 15 ml quantities prior to exposure to UV light.

Cells exposed to UV light were diluted in sterile saline, spread plated on Nutrient Agar (Difco), and incubated at 35° C until the

colonies were large enough to pick. Colonies were picked with sterile wooden toothpicks and transferred in identical patterns onto Mod IV medium, Mod V medium, and Nutrient Agar. These media were incubated at 35° C for 72 hours. Colonies which grew on Mod V medium and Nutrient Agar but not on Mod IV medium were considered to be nitrogen-fixation mutants. Growth on Mod V medium but not on Mod IV medium was taken as evidence that the cells were able to utilize nitrate as a nitrogen source, but were unable to fix atmospheric dinitrogen.

Mutants unable to fix atmospheric dinitrogen were designated Az3 nifa through Az3 nifc and were stored in the manner described for Bacillus cultures. A nitrogenase mutant of A. vinelandii was also obtained from H. L. Sadoff, Department of Microbiology, Michigan State University, East Lansing, MI, and designated A. vinelandii nif⁻. Further nitrogenase mutants and a revertant of a nitrognease mutant of A. vinelandii were obtained from W. J. Brill, Department of Bacteriology, University of Wisconsin, Madison, WI, and designated A. vinelandii n1, n2, and revertant. These mutants were tested to confirm their identity as azotobacters as described previously and were stored as described for Bacillus.

Reduced-slime mutants of Az3 were obtained by UV irradiation and were stored as described for azotobacters. These reduced-slime mutants were recognized by plating irradiated Az3 cultures on Mod III medium, as described above for nitrogen fixation mutants, and looking for "rough" colonies which did not exhibit slime when manipulated with an

inoculating needle. Reduced-slime mutants were designated Az3 sm1 through Az3 sm3.

Determination of Fermentation Optima

Determination of medium composition

Nitrogen Various nitrogen sources were tested using N-free salts medium (Mod IV) as a base. Atmospheric dinitrogen, potassium nitrate (Mallinckrodt), sodium nitrate (Fisher), ammonium chloride (Fisher), and Casamino Acids (Difco) were provided as nitrogen sources. Cultures of Az3 were grown in one-liter quantities in Fernbach flasks in these supplemented media at 30° C with shaking. A 1% inoculum of 30-hour old Az3 was used. Cells were harvested by centrifugation at 36 hours and cell mass and PHB content were determined. The presence of PHB was also monitored with NBS staining.

The amount of potassium nitrate in Mod IV basal medium was varied to determine the optimum amount of KNO_3 necessary for maximum PHB production and cell growth. Potassium nitrate was incorporated into Mod IV medium at levels of 0.00, 0.05, 0.07, 0.10, 0.12, and 0.15% (w/v). Az3 cells were grown in one-liter quantities in Fernbach flasks at 30° C as described above. Cells were harvested at 36 hours and cell mass and PHB content were determined. NBS staining was used to monitor PHB in the cells during growth.

Iron The iron content of the medium was varied and the growth of Az3 was monitored (cell mass and PHB levels were determined) using Mod IV medium as a base. Cells were grown in one-liter quantities in

Fernbach flasks at 30° C with shaking as described for nitrogen determinations. Cells were harvested at 36 hours. Iron was supplied as ferric chloride (Fisher) and ferric sulfate (Baker) at levels of 0.0025, 0.005, 0.01, and 0.1 grams per liter, in place of the normal iron levels in this medium. Mod IV medium without iron was used as a control.

Phosphate Phosphate content was varied in Mod III medium despite the fact that K_2HPO_4 provides an important buffer, particularly at high glucose levels. For this experiment, the buffer capacity of K_2HPO_4 was replaced by 0.2 M TRIS buffer (TRIZMA, Sigma). K_2HPO_4 content was varied to 0.00, 0.015, 0.05, 0.10, 0.17, 0.25, and 0.50 grams per liter. Cells (Az3 and Az19) were grown in 200 ml of modified Mod III medium in 500-ml Erlenmeyer flasks stoppered with gauze pads at 30° C with shaking. A 1% inoculum of 30-hour old cells grown in Mod IV medium was used. Cells were harvested at 36 hours and cell mass and PHB content were determined.

Calcium Calcium was varied as $CaCO_3$. $CaCO_3$ was included in Mod IV medium at levels of 10.0, 1.0, 0.5, 0.1, and 0.01 grams per liter. Mod IV medium without calcium was used as a control. The pH was monitored every two hours during the fermentation to insure that the pH did not fall below 7.2.

Az3 cells were grown in 1 liter quantities in Fernbach flasks stoppered with gauze pads at 30° C, and were harvested by centrifugation after 36 hours of incubation. A 1% inoculum of 30-hour old cells grown in Mod IV medium was used. The cell mass and PHB content of the harvested cells were determined.

Trace minerals The effect of trace minerals on PHB production in Az3 was determined by adding various levels of trace minerals, molasses, and other additives to 100 ml of Mod III medium (in place of the Mg, NaCl, and Mn concentrations normally found in this medium) in 250-ml Erlenmeyer flasks at 30° C with shaking. The base levels of minerals used were those suggested by previous researchers (Becking, 1962). A 0.1% inoculum of 30-hour old cells grown in Mod III medium was used. Deionized water was used in the preparation of the medium to avoid the addition of unwanted minerals, and all glassware used in this experiment was chemically clean. Chemical cleaning was accomplished by soaking glassware for four hours in chromic acid solution (10% w/v potassium chromate/concentrated sulfuric acid), rinsing five times in tap water, and soaking the glassware overnight in 1% w/v KOH in alcohol. The glassware was then rinsed five times in tap water and five times in distilled water, sealed with aluminum foil to keep out dust, and dried overnight at 50° C. Cells were harvested after 48 hours and cell mass and PHB content were determined.

To determine if water which was not as pure as the deionized or distilled waters normally used in medium preparation could supply a portion of the desired minerals, Mod IV medium without the Mg, Mo, or Mn levels normally present in this medium was prepared with tap water, well water (from Bremer County, IA), and deionized water. Mod IV with the normal mineral concentration and made with deionized water was used as a control. Cultures were grown in 100-ml of medium in 250-ml Erlenmeyer flasks stoppered with gauze pads. The incubation temperature was 30° C.

Cultures were incubated with shaking. A 0.01% inoculum of 30-hour old Az3 cells grown in Mod IV medium was used. After 48 hours of incubation, the cells were collected by centrifugation , and cell mass and PHB (as percent cell dry weight) were determined.

The various mineral supplements added and their concentrations are listed in Table 8.

TABLE 8. Minerals Added to Mod III Medium

Na_2MoO_4 (0.00001, 0.0001, 0.001, 0.01 g/l)
$MgSO_4 \cdot 7H_2O$ (0.002, 0.02, 0.2, 2.0 g/l)
NaCl (0.2 g/l)
$CuCl_2$ (0.01 g/l)
$CoCl_2$ (0.01 g/l)
ZnO (0.01 g/l)
$MnSO_4$ (0.0001, 0.001, 0.01 g/l)
Winogradsky's trace mineral solution (1 ml/l) (Daste et al., 1968)
Molasses (1 ml/l)
Ashed molasses (1 ml/l prior to ashing)
Mo + Mg + Mn (0.001, 0.02, 0.0001 g/l respectively)

pH The pH of Mod III medium was varied by adding hydrochloric acid or sodium hydroxide to the completed medium. The pH of the medium was varied to the levels of 4.5, 5.2, 5.8, 6.2, 6.8, 7.2, 7.4, 7.5, 7.8, and 8.0 (final pH after autoclaving).

Az3 cells (1% inoculum of 30-hour old cells) were added to 250 ml of pH-modified Mod III medium in 500-ml Erlenmeyer flasks. The cultures were incubated at 30° C with shaking. Cells were recovered by centrifugation after 48 hours. Cell mass and PHB content were

determined. The final pH (after growth) of the medium was also determined.

Aeration

The optimum aeration rate was approximated using a Bellco spin flask fermentor with attached oxygen valve as previously described, and with New Brunswick ten-liter fermentors with built-in air control valves.

Preliminary aeration studies were performed with an aeration manifold constructed by W. R. Lockhart, Department of Microbiology, ISU, and with a Beckman dissolved oxygen meter. These studies were performed with Az3 and Az19 cultures in 250-ml of Mod IV medium in 500-ml Erlenmeyer flasks.

Because the results of these preliminary tests were unsatisfactory, further preliminary aeration determinations were performed in the Bellco spin flask with 3 liters of Mod IV medium. Both Az3 and Az19 cultures were used with a 1% inoculum of 30-hour old cells grown in Mod IV medium. The fermentation was performed in a 30° C incubator at the highest rpm rate attainable with the magnetic stirrer motor/impeller combination used. The magnetic stirrer was insulated from the fermentation flask by several layers of paper toweling. The aeration rate was varied between 100 and 300 cc/min/liter in 50 cc/min/liter increments. The air was from laboratory compressed air lines and was prefiltered through glass wool, sterilized by passage through a 0.22 μm membrane filter (Millipore or Gelman) in an in-line filter holder

(Gelman), and humidified by bubbling the sterilized air through sterile deionized water. Cells were recovered after 36 hours of incubation by centrifugation (Sharples continuous-flow centrifuge, Sharples, Philadelphia, PA) at high speed. The cell mass and PHB content of the recovered cells was determined.

Final aeration studies were performed in New Brunswick fermentors at 30 and 35° C with the aeration rate varied between 500 and 3000 cc/minute with ten liters of Mod V medium. A 1% inoculum of 30-hour old Az3 cells grown in Mod V medium was used. The impeller drive of the fermentors was kept at setting 5 throughout these studies. Temperature was controlled with the built-in heating coil water bath assembly and by the use of a water bath cooling coil (Precision Scientific, Chicago, IL). Temperature was monitored by a TM Sentry Q recording thermometer as previously described. The aeration rate was adjusted by testing the flow rate with a water-displacement device (bubbling air into an inverted, submerged graduated cylinder). Air from laboratory lines was filtered and humidified as for the Bellco spin flask except that sterile cotton was used in place of glass wool. Az3 cells were recovered by centrifugation (Sharples) at 36 hours. Both cell mass and PHB content were determined.

Antifoams In aeration studies, severe foaming was sometimes encountered in the latter stages of the fermentation. Antifoam A emulsion (Sigma) or Foamkil spray (Nutritional Biochemicals) were added to control foaming.

To determine whether oils could be used as inexpensive antifoams, Mod V medium with a 1% inoculum of 30-hour old Az3 cells, grown in Mod V, was incubated at 30° C with shaking in 100-ml quantities in Erlenmeyer flasks stoppered with gauze pads. Antifoams were added to various flasks as follows: Foamkil, 15 second spray; antifoam emulsion A, 0.5 ml per liter; peanut oil, 1 ml per liter; soybean oil, 1 ml per liter; safflower oil, 1 ml per liter. Antifoams were sterilized by autoclaving prior to addition to the medium. Cells were harvested by centrifugation after 36 hours of incubation and the cell mass and PHB content of the isolated cells was determined.

Temperature effects To determine the optimum temperature for growth and PHB production, the producer strain, Az3, was inoculated into 100-ml quantities of Mod IV medium in 250-ml Erlenmeyer flasks. These flasks were placed in a waterbath (Precision Scientific Co.) to control temperature. The flasks were not shaken.

The temperature was monitored with a recording thermometer (as previously described). Temperature was varied to levels of 23, 24, 25, 27, 28, 30, 32, 33, 34, 35, 37, 39, and 40 degrees Celsius. The temperature control of the waterbath was accurate to within 1° C.

Cells were grown at the desired temperature for 36 hours, and collected by centrifugation. Cell mass (wet and dry weight), and PHB content (as percent cell dry weight), were determined as previously described. A 1.0% inoculum of 30-hour old cells grown in Mod III medium was used. The flasks were stoppered with gauze pads as previously

described.

Effect of Phenylacetic Acid on PHB Production

The method of Nuti et al. (1972) was used to determine the effect of phenylacetic acid and its analogs on cell mass and PHB production in Az3 and Az19. Phenylacetic acid (Krishell Laboratories, Portland, OR), phenyl acetate (Eastman), phenoxyacetic acid (Sigma), phenoxyethanol (Sigma) and phenol (Mallinckrodt) were added to 100-ml quantities of Mod I medium in 250-ml Erlenmeyer flasks. Phenylacetic acid and its analogs were sterilized by filtration (Millipore HAWP 0.45 µm membrane filter). After sterilization, these compounds were added to the media to obtain final concentrations of 100 ppm, 10 ppm, or 1 ppm. Mod I medium without phenylacetic acid and its analogs was used as a control. Az3 and Az19 were added to the prepared medium (1% inoculum of 30-hour old cells) and the cultures were incubated with shaking for 36 hours.

After incubation, cells were collected by centrifugation. Where noticeable growth had occurred, cell mass was determined. PHB content and the size and number of granules per cell was monitored by NBS staining.

Final Growth Curves

Growth studies in New Brunswick fermentors using Az3 in Mod V medium were performed using the optimal conditions determined in the previous studies. The reduced-slime mutant Az3 sm2 was also used to perform final growth studies in Mod V medium and New Brunswick fermentors.

Ten liters of Mod V medium in New Brunswick fermentors was inoculated with 1% Az3 or Az3 sm2 36-hour old cells grown in Mod V medium at the fermentation temperature. The inoculated fermentors were maintained at 33° C at an aeration rate of 150 cc air/liter of medium/minute at an impeller setting of 5. Samples were taken at intervals of one hour to four hours (depending upon stage of growth) and the optical density of the culture was determined at 660 nm. The total cell count (microscopic), viable cell count, cell morphology, pigment production, cell mass, and PHB content were determined as previously outlined. The amount of glucose remaining in the medium after cells had been removed by centrifugation was determined by the glucose oxidase/peroxidase method (Sigma diagnostic kit). The generation time (G), yield of biomass (Y_1), and yield of PHB (Y_2) was determined by the method of Akita et al. (1976). The ratio of cell dry weight/optical density at 660 nm was also determined for various samples in order to demonstrate the relationship between the two measurments.

Effect of Nitrogen Source on Encystment

The effect of various nitrogen sources on the encystment cycle of Azotobacter was determined by a modification of the method of Sadoff (1975). Az3 and Az19 cells were grown in Mod IV medium without vitamins and in Mod V medium without vitamins. A 0.1% inoculum of 36-hour old cells grown in Mod II medium was used. Both Az3 and Az19 were also grown in Mod V medium without vitamins where the potassium nitrate had been replaced with an equal amount of ammonium chloride. Both 1% and

2.3% glucose were used as carbon sources. Cells were grown in 100 ml of medium in 250-ml Erlenmeyer flasks stoppered with gauze pads. The cultures were incubated at 30° C for 150 hours. Samples were taken at 5 hour intervals and observed with phase-contrast microscopy and with the Azotobacter cyst stain of Vela and Wyss (1964). Both Az3 and Az19 cells were grown in Mod IV and Mod V medium supplemented with 0.2% n-butanol (Fisher) (added after the medium was autoclaved and cooled). These cells were grown for 150 hours and monitored for cyst formation as above.

PHB Production in Nitrogenase Mutants

Nitrogen-fixation mutants Az3 nifa, Az3 nifb, Az3 nifc, A. vinelandii nif- from H. L. Sadoff, and A. vinelandii n1, A. vinelandii n2, and A. vinelandii revertant from W. J. Brill were used in this experiment. These nitrogenase mutants were grown in Mod IV medium without vitamins, and containing no fixed nitrogen source or either nitrate or ammonium nitrogen, as described for the experiment on the effect of nitrogen on encystment. In addition, all media were inoculated with Az3 or A. vinelandii wild-type cells which were used as controls. Cultures were incubated with shaking at 30° C in 1000-ml quantities in Fernbach flasks stoppered with gauze pads. Cells were recovered by centrifugation after 36 hours and their cell mass and PHB content was determined. PHB content was monitored throughout growth by NBS staining. Cells were also examined with phase-contrast microscopy and cell motility was determined.

Statistical Calculations

All experiments were performed in duplicate or triplicate and the mean and standard deviation of the pooled data was determined by standard methods (Ostle and Mensing, 1975). Data which seemed far removed from similar data gained from other repetitions of the same experiment were subjected to the standard "T" test and were discarded if not valid at the 95% confidence level (Ostle and Mensing, 1975).

RESULTS

Isolation of PHB Producers

Members of the genus Azotobacter, which were isolated from Iowa or Florida soils, were tested for the production of PHB by staining with NBS or Sudan Black B. The isolates were rated from ++++ to + by visual estimation of the amount of intracellular PHB. Isolates rated + contained fewer fluorescent granules than the majority of the isolates when stained with NBS, but still exhibited some fluorescence. Isolates exhibiting no fluorescence upon staining with NBS were discarded. These isolates were also screened for the ability to produce an extracellular amylase, and were rated +++ to - based on the ability to produce PHB from starch-based media. The strains of Azotobacter retained for study, their ability to produce PHB as estimated visually, the ability to produce extracellular amylase, and the designation assigned to each strain are listed in Table 9.

These azotobacters were identified to the species level where this was possible, and the species name corresponding to each isolate is also listed in Table 9. The cultures of Bacillus, Dermatophilus, and Pseudomonas used in this study are also listed in Table 9, together with their PHB levels and ability to produce amylase.

Several members of the genus Azotobacter which produced large quantities of PHB and also produced an extracellular amylase were retained for further study. These strains were grown on nitrogen-free

TABLE 9. Bacterial Cultures Screened for PHB and Amylase Production

Culture	Identity[a]	PHB[b]	Amylase[c]	Culture	Identity	PHB	Amylase
Az1	C	++++	++	Az24	C	+++	++
Az2	C	+++	+	Az25	C	++	+
Az3	C	++++	+++	Az26	C	+	+
Az4	C	+	-	Az27	C	++	+
Az5	U	+	-	Az28	U	+	-
Az6	C	++	+	Az29	C	++	++
Az7	V	++	-	Az30	C	++	++
Az8	C	+	+	Az31	C	+	+
Az9	U	++	-	Az32	C	++++	+
Az10	C	+	++	Az33	V	++	-
Az11	C	+++	+	Az34	C	+++	+
Az12	C	++++	+	Az35	C	++++	+++
Az13	C	+	+	Az36	C	+	+
Az14	C	++++	++	Az37	C	+	++
Az15	C	+	+	Az38	C	++++	+
Az16	C	+	+	Az39	C	++	++
Az17	V	+++	-	Az40	C	+	+
Az18	C	++	+	*B. megaterium* KM		++	+
Az19	C	++++	+	*B. cereus* a		+++	-
Az20	C	+	+	*B. cereus* b		+++	-
Az21	C	+	+	*B. cereus* c		+++	-
Az22	C	+	+	*B. cereus* d		+++	-
Az23	C	++	++	*Pseudomonas* spp.		++	-
				Dermatophilus spp.		++	-

[a] C = *A. chroococcum*, V = *A. vinelandii*, U = unknown.

[b] PHB content assayed visually by NBS staining.

[c] Amylase assayed by zone diameter around colony on amylase agar. Amylase rated positive only if the colony exhibited a zone of clearing when grown on amylase agar and had PHB when grown on amylase agar.

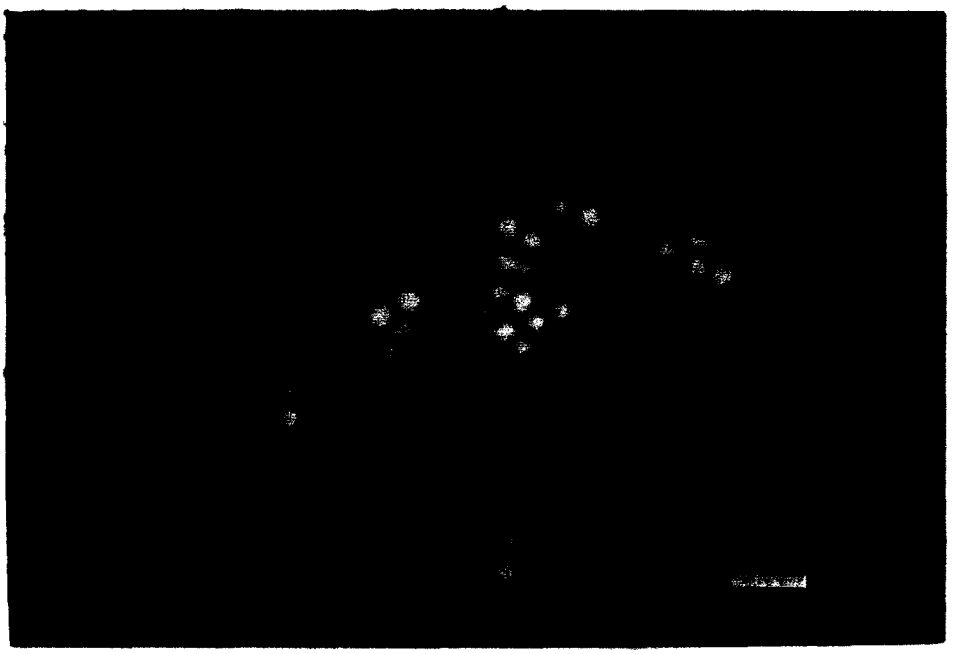
media and assayed to determine the amount of PHB produced as a percentage of cell dry weight. These data are presented in Table 10. PHB levels for growth in nitrogen-free broth with both glucose and starch as carbon sources are presented.

By far the majority of Azotobacter isolates are members of the species A. chroococcum. This species is apparently quite common in soil and may easily be recognized on solid nitrogen-free media by its large mucoid colonies and a tendency to produce a water-soluble brown pigment in the later stages of growth. When A. chroococcum cells are examined microscopically, the appearance of the brown pigment seems to correlate with the encystment of individual cells. The biochemical characteristics of the A.chroococcum isolated for this study are listed in Table 11. This species is a remarkably homogeneous group with few biochemical variants.

Staining of PHB Granules

When stained with Nile Blue Sulfate (NBS) as previously described, and examined under near-UV light in an epifluorescence microscope, PHB granules within individual cells fluoresced a bright orange. This fluorescence did not readily "quench" when exposed to exciting light for long periods of time (20 minutes or more). Individual granules were often visible within a cell. An example of PHB granules stained with NBS is presented in Figure 11.

When NBS was compared to Sudan Black B, it was found that many more



A. chroococcum Az3, 72-hour old cells grown on Mod I medium, examined under epifluorescent near-UV light and transmitted light. Bar = 3 μm

FIGURE 11. PHB Granules Stained with Nile Blue A

TABLE 10. Production of PHB in Media Containing Different Carbohydrates

Strain	% PHB in glucose	% PHB in starch
Az1	66	60
Az3	72	67
Az12	70	66
Az14	70	64
Az19	68	63
Az23	65	59
Az24	52	46
Az29	55	40
Az32	50	44
Az34	51	43
Az35	70	67
Az38	65	58
Average standard deviation = 1.02%		

[a]2% carbohydrate in Mod III medium, in place of the normal carbohydrate present in this medium. Grown at 30° C in Fernbach flasks with shaking. A 0.01% inoculum was used and the cells were harvested at 36 hours post-inoculation. PHB was isolated by hypochlorite digestion.

PHB granules in identical cultures stained with NBS than with Sudan Black B. Sudan Black B appeared to decolorize readily when exposed to xylol, the recommended decolorizing agent. NBS did not wash out of PHB granules when decolorized for up to 10 minutes in 8% aqueous acetic acid. In vitro staining of glycogen and the staining of metachromatic (polyphosphate) granules in Corynebacterium diphtheriae determined that these compounds did not fluoresce when stained with NBS. Tributyrin did fluoresce when stained with NBS but the fluorescence of this lipid was a

TABLE 11. Biochemical Characteristics of A. chroococcum Isolates

	Az1	Az2	Az12	Az14	Az19	Az32	Az35
Fluorescent pigment	-	-	-	-	-	-	-
Brown pigment	+	+	+	+	+	+	+
Amylase	+	+	+	+	+	+	+
Growth on mannitol[a]	+	+	+	+	+	+	+
Growth on ethanol[a]	+	+	+	+	+	+	+
Growth on rhamnose[a]	-	-	-	-	-	-	-
Motility[b]	+	+	-	+	+	+	-
Slime produced	+	+	+	+	+	+	+
Cysts formed[c]	+	+	+	+	-	+	-
Catalase	+	+	+	+	+	+	+

[a]1% carbohydrate in Mod I medium in place of the normal carbohydrate content of that medium.

[b]Motility was determined by observation of wet-mounts with phase-contrast microscopy.

[c]Determined by the Azotobacter cyst stain after 96 hours of growth on Mod I medium.

light yellow, similar to that noted when immersion oil contacted stained cells, and is easily distinguished from fluorescent PHB granules. Cells grown under conditions unfavorable for PHB accumulation (growth on Nutrient Agar for azotobacters) did not exhibit fluorescent granules when stained with NBS.

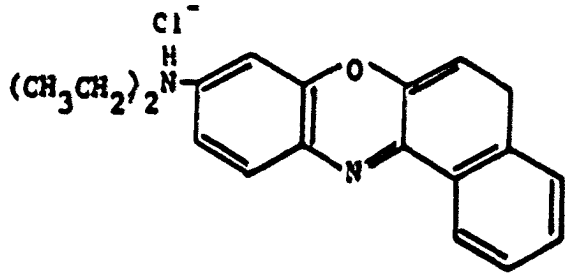
If heat fixed cells on slides were subjected to solvent extraction prior to NBS staining as previously described, those cells treated with PHB solvents (chloroform and dichloromethane) and then stained did not

exhibit fluorescent granules while those cells treated with other lipid solvents (benzene, ether) did. This tends to confirm that the stained granules observed when Azotobacter cells are stained with NBS are indeed PHB. The presence of PHB in these cells was also confirmed by hypochlorite extraction and degradation to crotonic acid. Extracted, purified PHB did not stain well with NBS.

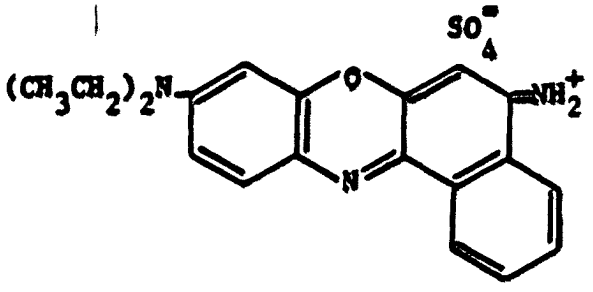
Such extraction steps must be performed prior to staining with NBS as the solvents will extract the dye from PHB granules. These extractions were performed with similar results in Pseudomonas, Dermatophilus, and Bacillus.

When stained Azotobacter cells were examined with a fluorimeter, the observed fluorescence detected at 550 nm was strongest at an excitation wavelength of 362 nm. The Nikon blue excitation method used for microscopic observation has an excitation wavelength of approximately 460 nm. Two peaks of detected fluorescence were observed, one at 550 nm and one at 430 nm. These characteristics agree with the published characteristics of Nile Pink, the oxazone form of Nile Blue (Lille, 1977). Thus, it is probable that Nile Pink is the actual staining agent of PHB granules in this method. Published methods (Thompson, 1966; Lille, 1977) call for the production of Nile Pink by the acid reflux of Nile Blue. It is apparent that there is enough of the oxazone form of Nile Blue present in aqueous mixtures so that acid reflux is not needed. The dye Meldola Blue (Figure 12) is a close chemical relative of Nile Blue/Nile Pink. When Meldola Blue was substituted for Nile Blue in this

staining procedure no fluorescence of PHB granules was detected. This would seem to indicate that a small portion of the molecule is responsible for either the fluorescent staining or the adsorption of the dye to PHB. Meldola Blue is known to be a fluorescent stain of lipids (Ferrans, 1963).



Meldola Blue



Nile Blue A

FIGURE 12. Structure of Nile Blue A and Meldola Blue

Nile Blue staining was used as a quantitative assay for PHB by using the fluorimeter to measure the observed fluorescence of bacterial samples at 550 nm and comparing this fluorescence to the amount of PHB detected by chemical isolation and assay of the polymer. PHB was chemically isolated by hypochlorite degradation. These data are presented in Figure 13. The assay was very sensitive to delays in obtaining fluorometric readings after stain had been added to the cells.

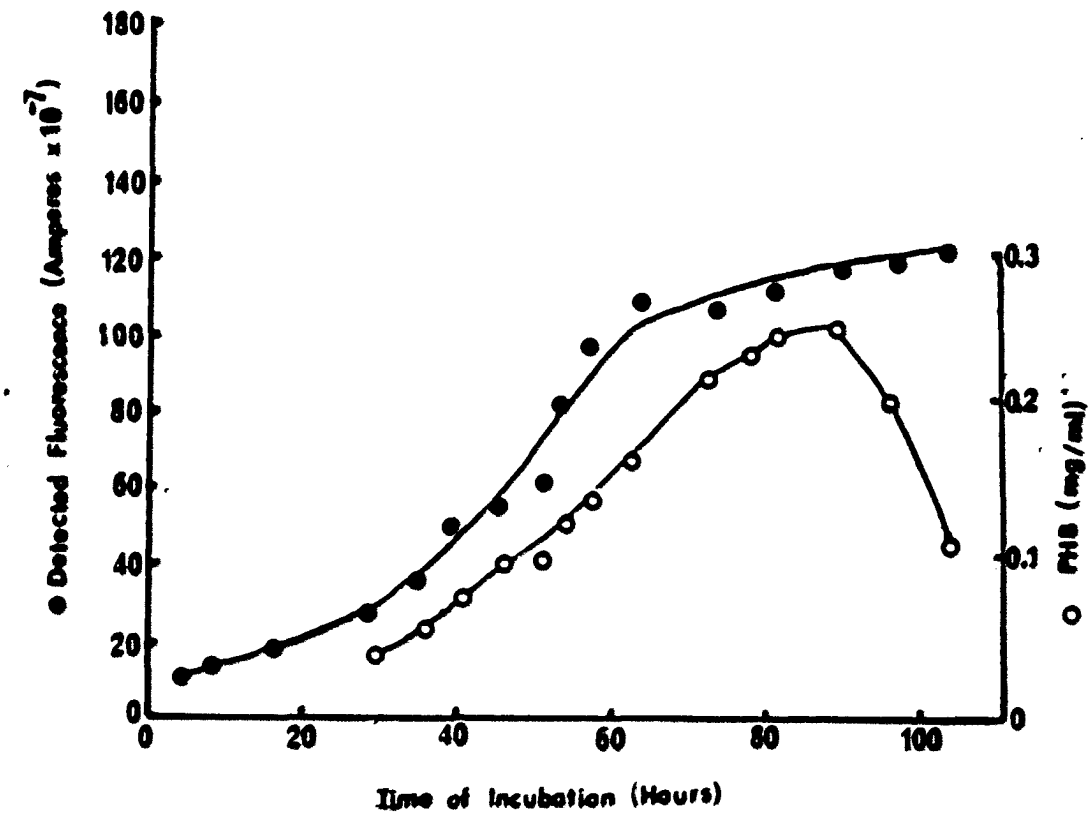


FIGURE 13. Assay of PHB by NBS Staining and Fluorimetry

Delays of less than 20 minutes often resulted in a loss of over 80% of the observed fluorescence as compared to samples read immediately after NBS addition. When the assayed cells were examined with an epifluorescence microscope, no fluorescing structures other than PHB granules were observed, and PHB granules seemed to fluoresce normally in hour-old samples.

NBS was unable to stain PHB in chromatographs performed on glass fiber filter paper, and was unable to stain PHB in vitro. Chromatographs on Whatman 3MM paper could not be stained with NBS due to the generalized yellow fluorescence of the paper stained with NBS. Sudan Black B did not perform well as a chromatograph stain as it did not decolorize well, and stained the paper. On glass-fiber paper, chromatographs stained with Sudan Black B appeared to show that PHB did not migrate with the solvents, but stayed at the origin. Small spots of sudanophilic material may not have been noticed due to the poor decolorization of Sudan Black B.

A comparison of the data in Table 9 with the data in Table 10 indicates that while it is possible to estimate the amount of PHB in a cell by estimating the number of fluorescent granules visible, this method is not as accurate as isolation and chemical assay of the polymer. Several strains which appeared visually to have less PHB than strains rated +++ or ++++ have more PHB as a percentage of cell dry weight than do the +++ or ++++ strains. This may be due to the difficulty in estimating the number of fluorescent granules in cells

which are as tightly packed with PHB as are many azotobacters. It may be concluded that while visual estimation of the number of PHB granules in a cell by microscopic observation is usable as a qualitative PHB assay, it is no substitute for isolation and assay of the polymer when accurate quantitative data are desired.

Phosphine GN also stains PHB granules and granules so stained exhibit a white fluorescence under UV or near UV light. While Phosphine GN may be a superior PHB stain in a class with NBS (on the basis of preliminary screening) the extreme carcinogenicity of this compound precluded its further use in this study.

Preliminary Growth Studies

Growth curves

To establish methodology and sampling times for further studies, the strains of Azotobacter found to produce the highest levels of PHB (as percent cell dry weight) were used to determine preliminary growth curves. Four strains of Azotobacter chroococcum were selected for the ability to produce large amounts of PHB (over 69% of cell dry weight) in glucose, as well as PHB production (over 63% cell dry weight) in starch. These strains, Az12, Az14, Az35, and particularly Az3, the highest producer under both conditions, were used to determine fermentation optima. PHB (as percent cell dry weight and as total recovered weight), optical density, viable cell count, and total cell count were determined. Growth curves for Az3 and PHB levels in this organism are presented in

Figure 14 and Figure 15.

Within 96 hours after inoculation, about 38% of the cells (by visual estimation) had formed cysts. Cyst formation was determined by the use of Azotobacter cyst stain. A definite intine and exine similar to that described by Sadoff (1975) was noted. PHB declines rapidly to about 20% of cell dry weight with the onset of cyst formation. Microscopic observation of cell populations as they encyst shows that many cells do not completely encyst, but enter "abortive encystment". Cells which enter "abortive encystment" do not complete the formation of cyst intine or exine, and are larger than fully encysted cells.

PHB production with various carbohydrate sources

PHB production with various carbohydrate sources was determined in nitrogen-free medium (mineral salts base) containing 2% carbohydrate. The amount of PHB produced by Az3 after 36 hours (the end of log phase) in each of the various carbohydrates is presented in Table 12.

The amount of PHB obtained (and reported as percent cell dry weight) was greatest with glucose, sucrose, or fructose as carbon sources. The total cell mass after 36 hours was also the greatest when these carbon sources were used. PHB was not produced when casein was incorporated in the medium, nor was PHB produced in nutrient broth or on the cornsteep liquor based medium used for the growth of Bacillus.

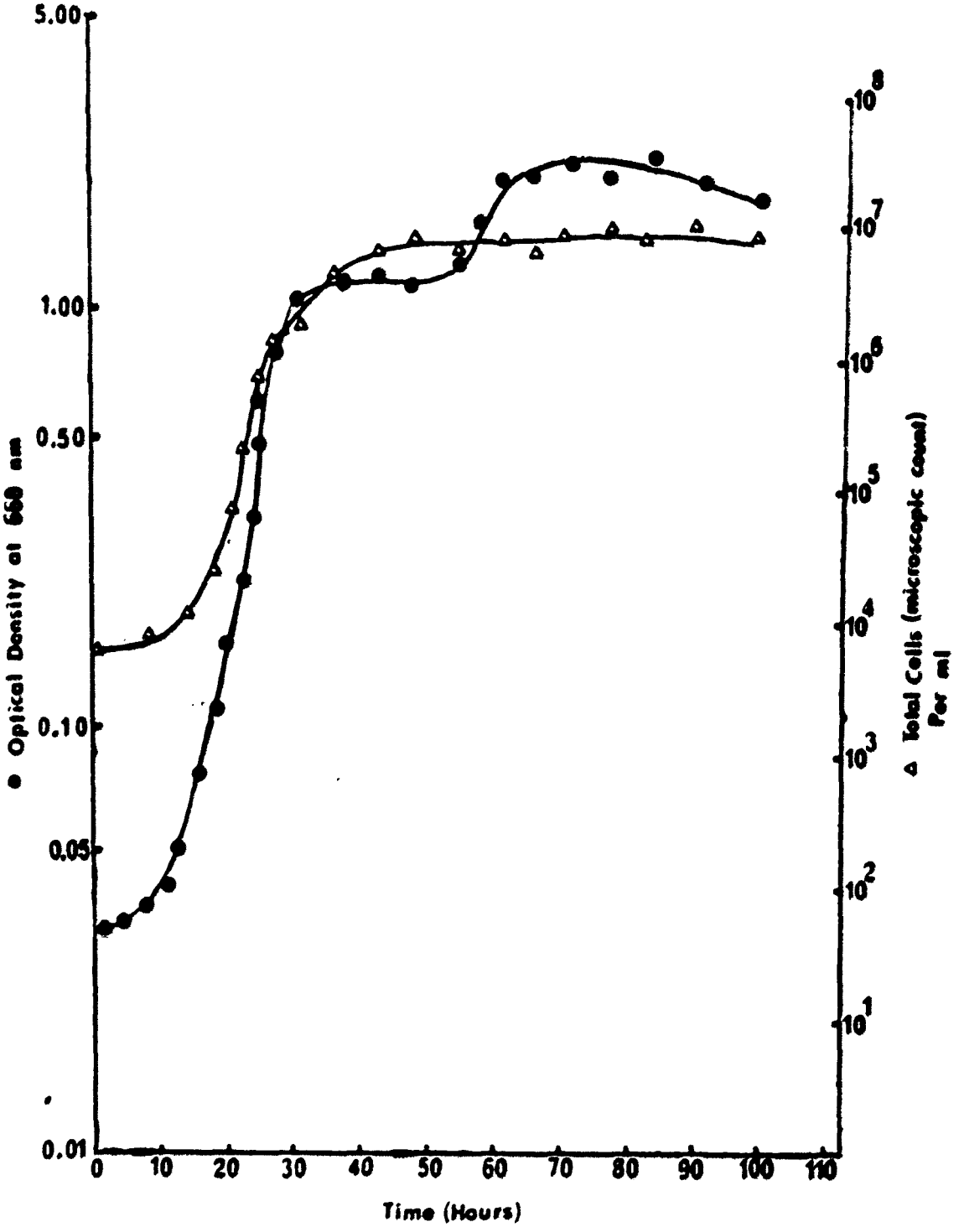


FIGURE 14. Growth of Az3

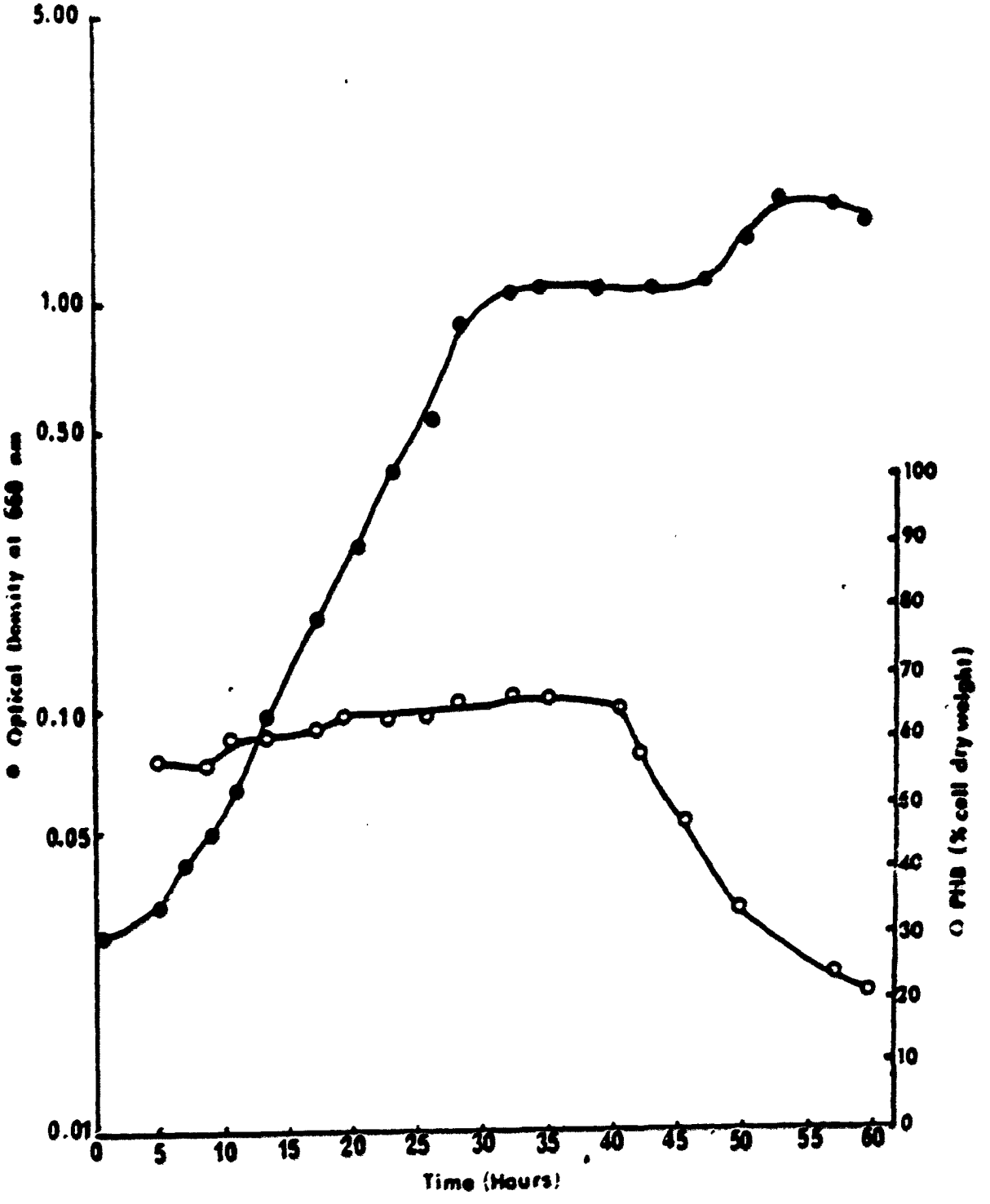


FIGURE 15. PHB levels in Az3

TABLE 12. PHB Content of Az3 vs. Carbon Source

Carbon source (2%)[a]	PHB % Cell Dry Weight	Cell Dry Weight (g/l)
Glucose	62	4.51
Sucrose	60	1.88
Fructose	58	0.96
Soluble Corn Starch	51	0.51
Ethanol	50	0.46
Maltose	48	0.44
Galactose	47	0.44
Inositol	46	0.43
Mannitol	44	0.39
Trehalose	31	0.33

[a]Carbon sources tested at 2% (w/v) levels in Mod III medium in place of normal carbohydrate levels in that medium. Az3 cells were grown in 1 liter quantities at 30° C with shaking, and were harvested at 36 hours post-inoculation. A 0.1% inoculum was used.

Growth curves with 1% glucose, sucrose, fructose, or soluble starch as carbon sources were performed with Az3 cells in one liter quantities in Fernbach flasks. The soluble starch used was prepared from cornstarch by acid hydrolysis. Cell growth was monitored by optical density. These data are presented in Figure 16.

PHB levels were monitored by NBS staining throughout these growth studies. PHB granules were noted in large numbers in the vast majority of cells throughout the growth period. PHB levels (as percent cell dry weight) at the end of log growth phase correspond to those noted for these carbohydrate sources in Table 12.

Glucose concentration

To determine the optimum glucose concentration for PHB production, glucose concentration in nitrogen-free media was varied and cell mass and PHB concentration were determined at each carbohydrate level. Glucose was chosen because of its inexpensiveness and good growth characteristics. Samples were taken at both 36 hours, the end of log phase, and at 60 hours, well into stationary phase. These data are presented in Figure 17.

Some PHB may be noted microscopically in encysted cells (20% of cell dry weight when assayed in 190-hour-old cultures grown in 1% glucose), indicating that all PHB in the pre-cyst cells is not used in the process of encystment. The process of encystment will be discussed in greater detail later in this work.

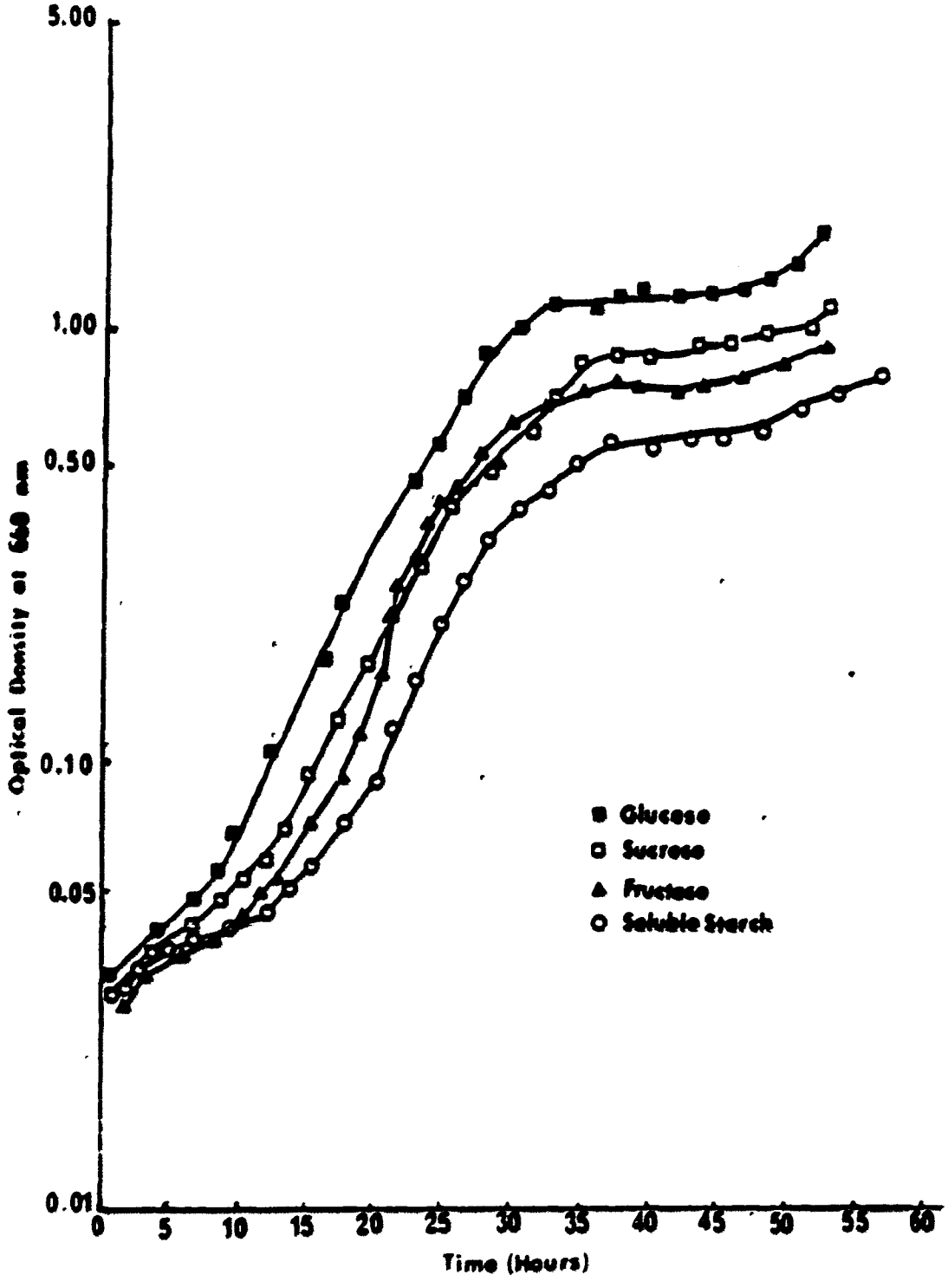


FIGURE 16. Growth of Az3 vs Carbohydrate Source

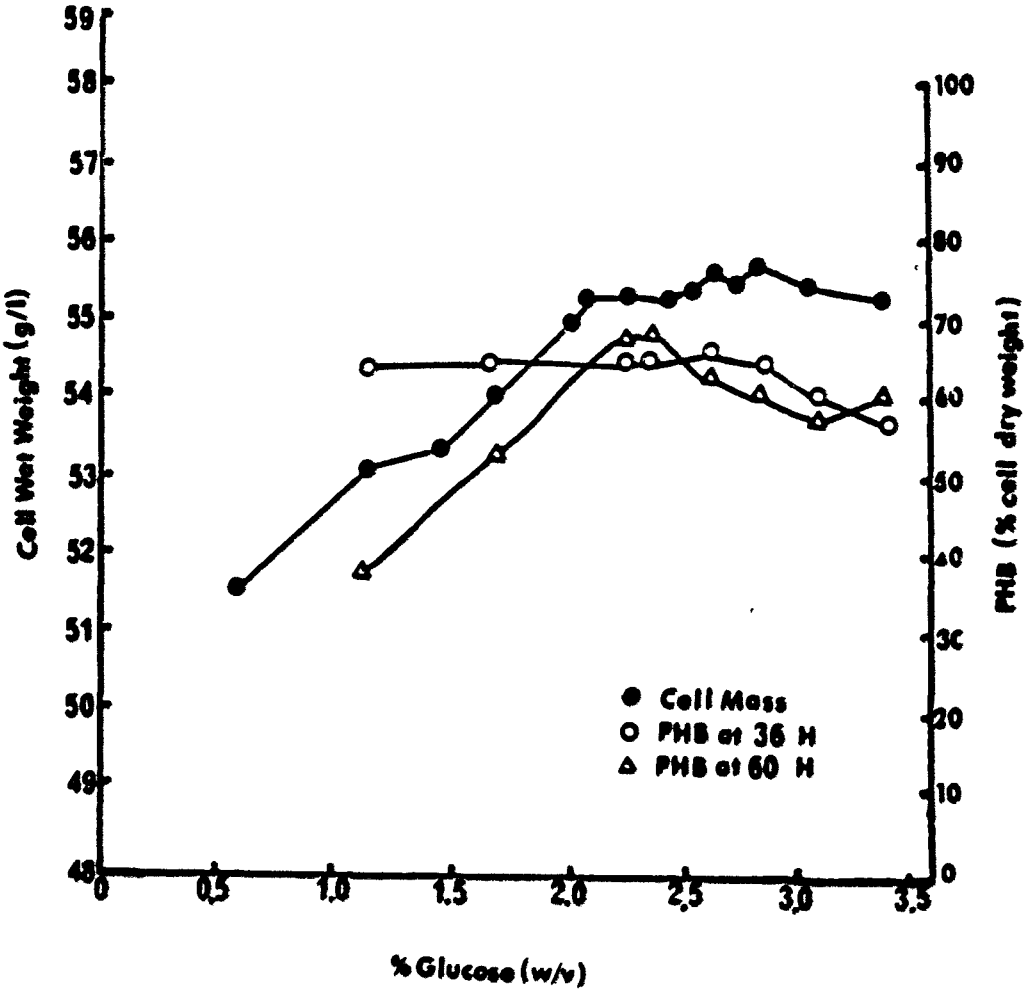


FIGURE 17. Effect of Glucose Concentration on Cell Mass and PHB Concentration

Various nitrogen sources were tested using a nitrogen-free salts medium (Mod IV) as a basal medium. The effect of nitrogen source and content on cell mass and PHB percentage of cell dry weight was determined with 36-hour old Az3 in Mod IV medium at 30° C. These data are presented in Table 13.

Iron content

The iron content of nitrogen-free Mod II medium was varied and the growth of Az3 was monitored. Both $FeCl_3$ and $FeSO_4$ were used as iron sources. With low iron content (at or below 0.0025 g/l) A. vinelandii produced a green, water soluble pigment evident on solid media. These data are presented in Table 14.

Effect of phosphate

Phosphate content was varied in standard Mod III medium, despite the fact that K_2HPO_4 provides an important buffer against acid produced during the growth of Az3. For this experiment, the buffer capacity was replaced by TRIS buffer. PHB as a percentage of cell dry weight and cell mass at various levels of phosphate are presented in Figure 18.

The effect of calcium on the growth of Az3

Calcium was varied in standard nitrogen-free media as $CaCO_3$. It should be noted that $CaCO_3$ has acid buffering effects in addition to supplying calcium. Calcium carbonate does not enter solution

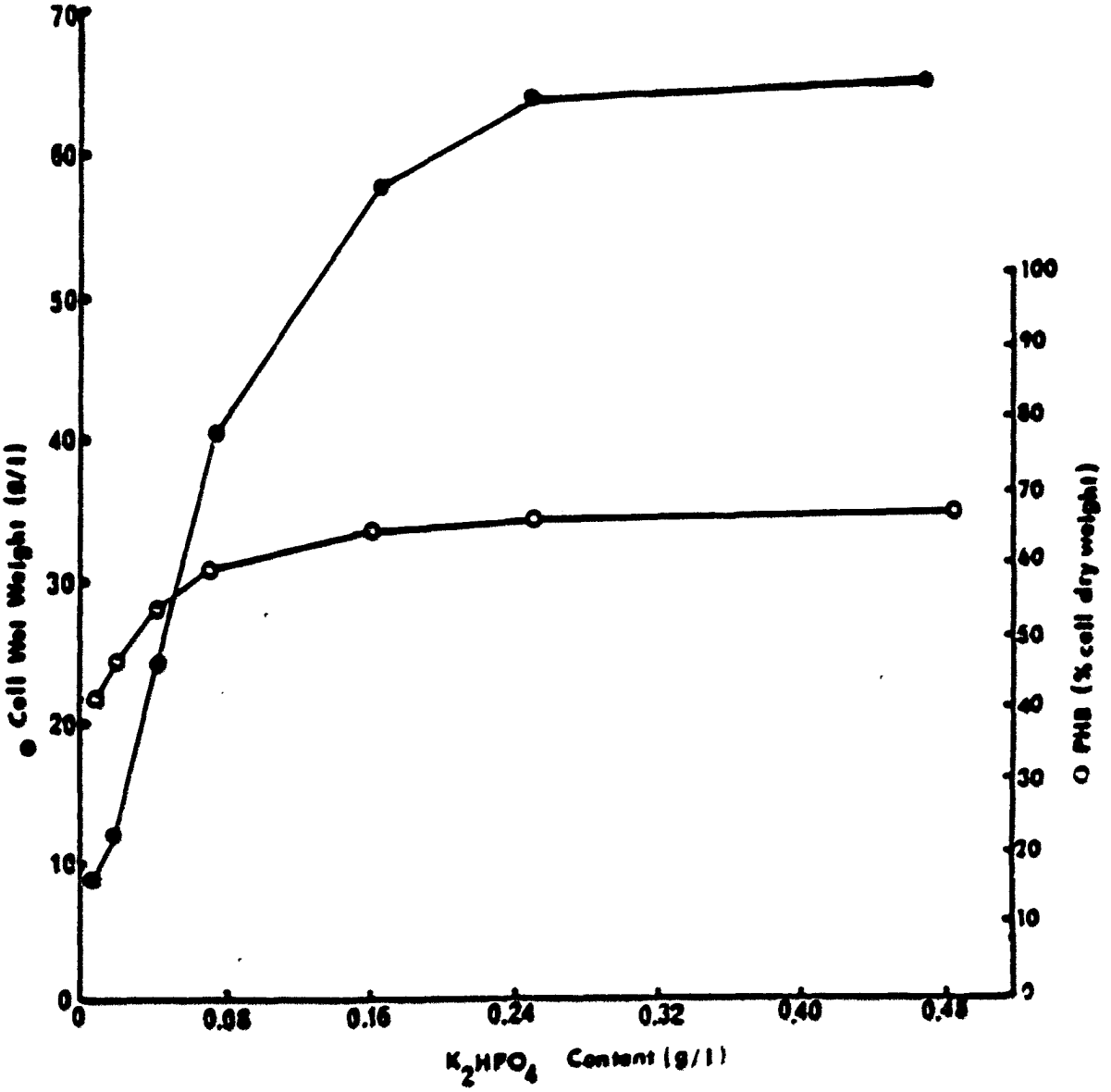


FIGURE 18. Effect of Phosphate Content on the Growth of Az3

completely, and remains as a precipitate in the medium unless acid is added. Data on the effect of varying calcium concentration are found in Table 15.

A tenth the amount of calcium carbonate normally found in Norris' nitrogen-free medium (Durand et al., 1982) does not affect the growth of Az3 or any other Azotobacter spp. used in this study. The PHB content of these cells is also unaffected. No serious decline in cell mass is seen until less than 0.01 g/l of calcium carbonate is reached.

As calcium carbonate is present in high amounts for its buffer capacity, Mod V medium normally contains excess calcium. In this experiment, the phosphate buffer capacity was increased to 2.0 g/l K_2HPO_4 and the pH was monitored throughout the growth of the organism to insure that the lack of buffer capacity did not affect the results. In no case did the final pH of the spent medium fall below 7.1.

Trace minerals

In preliminary experiments, molasses was added to Mod II medium which was used to grow Az3 and Az19 cultures. When molasses was added to this medium, an increase in cell yield was noted upon the harvest of the cells. Therefore, an attempt was made to determine which constituents of molasses were increasing the growth of Az3. Since xylose is often found in various molasseses (Zabriskie et al., 1980), all Azotobacter cultures, including Az3 and Az19, were screened for the ability to use xylose as a sole carbon source and for the ability to cometabolize

TABLE 13. Effect of Nitrogen Source on Cell Mass and PHB Content

Medium[a]	Approximate % N	Cell Dry Weight (g/l)	PHB Content (% cell dry weight)
Nitrogen-free	0	3.5	73
0.1% KNO_3	0.0138	5.8	77
0.01% $NaNO_3$	0.0165	5.6	76
0.01% NH_4Cl	0.0260	5.6	12
0.01% Casamino acids[b]	0.0100	4.7	14
0.05% KNO_3	0.0069	4.4	75
0.07% KNO_3	0.0097	4.9	77
0.10% KNO_3	0.0138	5.8	77
0.12% KNO_3	0.0166	5.9	77
0.15% KNO_3	0.0207	5.8	76

[a]Mod IV medium used as a basal medium for nitrogen addition.

[b]% N based on 10% total nitrogen in casamino acids (from Difco analysis).

xylose. None of these strains were able to utilize xylose as a sole carbon source or to cometabolize xylose with glucose (as measured by increased cell mass). Repeated attempts were made to isolate an Azotobacter strain capable of utilizing xylose as a sole carbon source. Although various enrichment techniques were used in this attempt, no xylose-utilizing Azotobacter was found. An azotobacter-like organism capable of utilizing xylose (and also cellulose, hemicellulose, and starch) as sole carbon sources has been reported (Caceres and Martinez-

TABLE 14. Effect of Iron on the Growth of Az3

Iron Type and Content[a]	Iron % Fe	Cell Dry Weight (g/l)	PHB % Cell Dry Weight
No Fe	0	0.9	75
$FeCl_3 \cdot 6H_2O$ 0.0025 g/l	0.00005	3.0	77
$FeCl_3 \cdot 6H_2O$ 0.005 g/l	0.000103	3.6	78
$FeCl_3 \cdot 6H_2O$ 0.01 g/l	0.000207	3.5	75
$FeCl_3 \cdot 6H_2O$ 0.1 g/l	0.00207	3.3	74
$FeSO_4 \cdot 7H_2O$ 0.0025 g/l	0.00005	2.4	76
$FeSO_4 \cdot 7H_2O$ 0.005 g/l	0.0001	2.8	77
$FeSO_4 \cdot 7H_2O$ 0.01 g/l	0.0002	3.0	78
$FeSO_4 \cdot 7H_2O$ 0.1 g/l	0.0020	2.9	77
Standard Deviation	=	0.1 g	1.5 %

[a]Mod IV medium was used as a basal medium. Iron contents shown are in place of the normal iron content of this medium. Cells were grown in one-liter quantities with shaking and were harvested at 36 hours post-inoculation. A 1.0% inoculum was used.

Peinado, 1980), although the identity of this isolate is uncertain (P. Caceres, Department of Microbiology, Faculty of Sciences, Badajoz, Spain, personal communication, 1981).

Sucrose and fructose are found in molasses (Zabriskie et al., 1980), but the addition of sucrose and fructose to media already containing 2.5% glucose did not result in increased cell mass, while the addition of molasses did. Therefore, various trace minerals were added in small amounts to standard nitrogen-free media in order to determine if the growth-stimulating effects of molasses were due to the addition of one or more of these trace minerals. Molasses and ashed molasses were added as a control. The data obtained in this study are listed in Table 16.

TABLE 15. Effect of Calcium on the Growth of Az3

Calcium Type and Content[a]	Calcium % Ca	Cell Dry Weight (g/l)	PHB % Cell Dry Weight
No Ca	0	0.81	60
$CaCO_3$ 10.0 g/l	0.4	2.82	67
$CaCO_3$ 1.0 g/l	0.04	2.86	66
$CaCO_3$ 0.5 g/l	0.02	2.76	68
$CaCO_3$ 0.1 g/l	0.004	2.51	67
$CaCO_3$ 0.01 g/l	0.0004	1.82	68
Standard Deviation	=	0.08 g/l	1.4%

[a]Mod IV medium was used as a basal medium. Calcium contents shown are in place of the normal calcium content of this medium. Cells were grown in one liter quantities with shaking and harvested at 36 hours post-inoculation. A 1.0% inoculum was used.

In this study, the nitrogen-free medium (Mod III) used for the determination of trace mineral effects did not contain the magnesium, manganese, or sodium chloride normally found in the various media designated as Mod media.

Many of these minerals can probaby be supplied by using tap water

TABLE 16. Effect of Trace Minerals on the Growth of Az3

Supplement[a]	Concentration per liter		Cell Dry Weight (g/l)	PHB (% cell dry weight)
None	0.000		2.5	66
Mo (Na_2MoO_4)	0.01	g	2.6	69
Mo	0.001	g	2.6	68
Mo	0.0001	g	2.5	68
Mo	0.00001	g	2.5	65
$MgSO_4 \cdot 7H_2O$	2.0	g	1.5	65
Mg	0.2	g	2.8	68
Mg	0.02	g	2.6	66
Mg	0.002	g	2.6	65
NaCl	0.2	g	2.5	66
$CuCl_2$	0.01	g	2.4	65
$CoCl_2$	0.01	g	2.3	64
$MnSO_4 \cdot 4H_2O$	0.01	g	2.5	66
Mn	0.001	g	2.6	65
Mn	0.0001	g	2.4	60
ZnO	0.01	g	2.4	65
Trace Mineral Solution	1.0	ml	2.9	67
Molasses	1.0	ml	3.1	68
Molasses (ashed)	1.0	ml	2.8	65
Mo	0.001	g		
Mg	0.2	g	2.7	67
Mn	0.001	g		

[a]Mod III medium was used as a basal medium for this experiment. The mineral additions shown are in place of the mineral concentrations normally found in this medium. Cells were grown in 100 ml quantities in 250-ml Erlenmeyer flasks with shaking and harvested at 48 hours post inoculation. A 0.01% inoculum was used.

instead of deionized or distilled water for media preparation. When Mod IV medium was prepared using untreated deep well water instead of deionized water, and the trace minerals were omitted from the Mod IV medium made with well water, the cell mass and PHB yields of both media were higher, although not equivalent to Mod IV with minerals (see Table 17).

TABLE 17. Cell and PHB Yields in Mod IV with Various Water Sources

Water Type [a]	Cell Mass [b] (g/l)	PHB [c] (% cell dry weight)
Deionized	24.3	67
Tap Water	25.8	66
Well Water	26.2	67
Mod IV with minerals [d]	31.1	68

[a]Mod IV medium without the Mg, Mo, or Mn normally found in this medium. Cells were grown in 100 ml quantities with shaking at 30° C., and harvested at 48 hours. A 0.01% inoculum was used.

[b]Standard deviation = 0.7 g.

[c]Standard deviation = 1.7%.

[d]Made with deionized water.

Effect of pH

The optimum pH levels for the accumulation of cell mass and the production of PHB by Az3 cells were determined by varying

the pH of Mod III medium. These data are presented in Figure 19.

The cell mass attained at 48 hours is fairly constant between pH 6.8 and 8.0. Az3 is sensitive to acidic conditions, and does indeed produce some acid from glucose (albeit very slowly). The production of acid from glucose was determined by monitoring the pH of Mod III medium throughout growth. When Az3 was grown in 200 ml of Mod III medium in 500-ml Erlenmeyer flasks at 30° C with shaking, the pH of the medium dropped an average of 0.3 pH units over a 48 hour period. Over periods of one week, a pH drop could be detected on solid media (nitrogen-free) when brom thymol blue was added as a pH indicator. The pH of solid media was determined after solidification by the use of a flat-surface pH electrode.

Effect of temperature

The temperature optimum was determined in flasks in a waterbath without shaking. The temperature of the waterbath was monitored with a recording thermometer and the waterbath was found to cycle through a 1° C range over a period of approximately 20 minutes. Thus, temperature gradations below 1° C were not attempted. For the same reason, the data presented below are not considered to be accurate at gradations finer than 1° C. These data are presented in Figure 20.

The lack of shaking in this study resulted in a somewhat lower cell yield as compared to other experiments at equivalent temperatures where shaking or other aeration methods were used. Growth occurred at

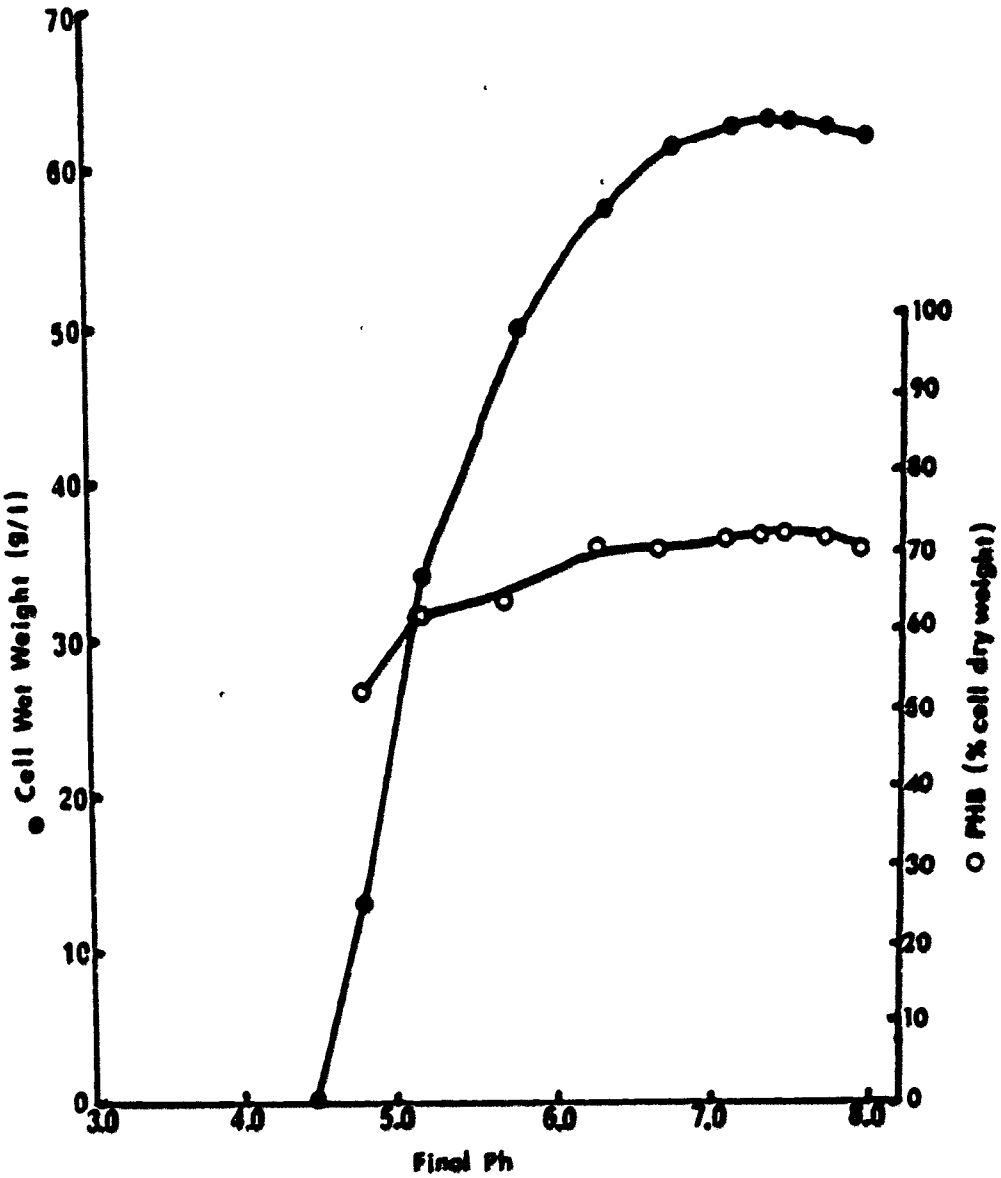


FIGURE 19. The Effect of pH on PHB Production and Growth in Az3

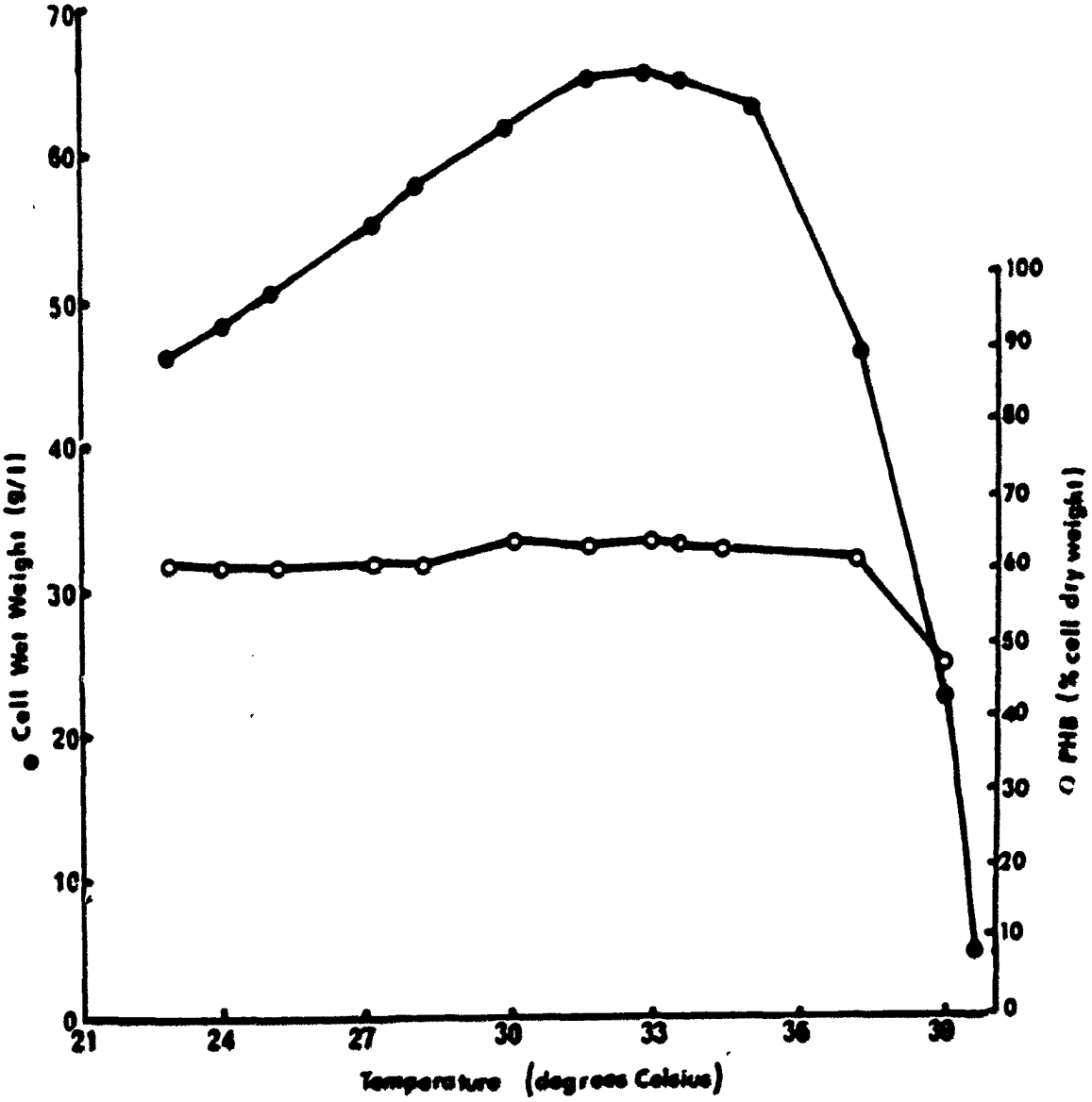


FIGURE 20. Effect of Temperature on Growth and PHB production in Az3

temperatures as high as 42° C but no growth occurred at 45° C. Growth between 42 and 45° C was uncertain, being occasionally detected, and occasionally absent. Perhaps growth proceded at a slow rate in this temperature range.

Growth at 37° C is very good, however the temperature optimum for both growth and PHB production would appear to be 33° C. This temperature optimum is higher than that previously reported for Azotobacter (Johnstone, 1974). This higher temperature is of importance as a higher growth rate is obtained at this temperature (see Final Growth Studies) and because of the expense necessary to cool large-scale fermentations.

The data presented in this experiment were obtained by sampling 72 hours after inoculation. This increased incubation time, as opposed to the normal sampling time at 36-38 hours (end of log phase) was necessary due to the slower growth of the organisms without shaking. Seventy-two hours was determined to be the approximate end of log phase at 30° C without shaking by a preliminary growth study.

Initial screening of many of the original Azotobacter isolates for the ability to grow at 37° C on solid nitrogen-free media (Mod III) indicated that many of these strains grow poorly at this temperature, although some growth is usually apparent after 48 hours.

Further refinement of temperature optima was performed in large New

Brunswick fermentors with up to 10 liters of medium and aeration. These data are discussed later (see Final Growth Studies).

Aeration

Aeration is of great importance in the growth of azotobacters. The optimum aeration rate was approximated, despite the lack of suitable equipment. The use of a dissolved oxygen meter to monitor effective aeration was unsuccessful due to the extreme variation in meter readings under standard conditions.

The use of an aeration manifold with various glass spargers produced no useful data. The aeration manifold was unsuccessful due to the inability to reproduce aeration rates from day to day even when the meter readings were identical. Therefore, preliminary aeration studies were carried out in a Bellco spin flask, and final aeration studies were performed in New Brunswick fermentors. The data obtained from preliminary aeration studies in the Bellco spin flask with Az3 are presented in Figure 21.

Cell mass increases when the culture is aerated to 50 cc/min/liter of medium. It would appear that the maximum necessary aeration rate is reached before this point. However, aeration cannot be reliably measured below this level with the oxygen flow meter adapted to the Bellco spin flask. A decline in PHB content (as a percentage of cell weight) is noticeable at higher aeration rates. PHB content declines at these increased aeration rates before a decline in total cell mass can

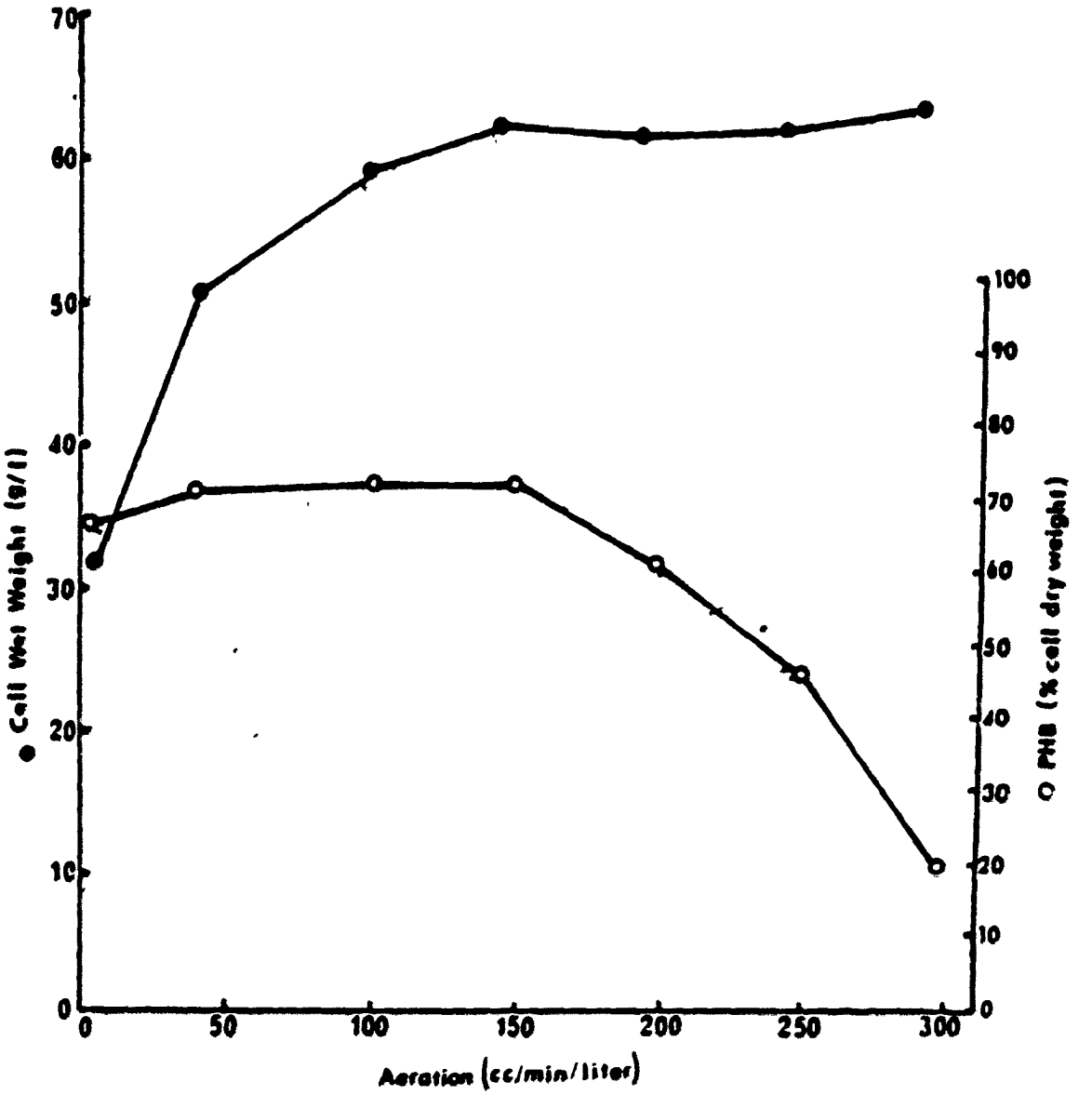


FIGURE 21. Effect of Aeration Rate on Az3

be detected. Examination of individual cells by phase-contrast microscopy and NBS staining confirmed that fewer PHB granules were visible in the cells.

Antibiotic Sensitivity

The Antibiotic susceptibility of Az3 by the standard FDA disk-diffusion method was performed for the purpose of determining which antibiotics could be used to aid in the elimination of contaminants in stock cultures, as well as to prevent contamination in fermentations. Contamination of working cultures was a recurring problem due to the large amount of slime produced by this organism. This slime had the effect of binding contaminating organisms (usually Pseudomonas) to clumps of Azotobacter, making the separation of the two organisms by standard microbiological methods difficult. The antibiotic susceptibility spectrum of Az3 is presented in Table 18.

Although Az3 and Az7 were resistant to penicillin G, distinct morphological effects could be noted in cells exposed to this antibiotic. Large, ribbon-shaped cells were often noted when penicillin G was added to Az3 cultures. These large cells were from 10 to 30 micrometers in length and from 2 to 5 micrometers in diameter. The large cells contained numerous PHB granules. No antibiotic to which Az3 or Az7 was resistant affected the production of slime by these organisms.

TABLE 18. Antibiotic Sensitivity of Az3 (Az7 same)

Antibiotic	Disk Content (µg)	Reaction
Erythromycin	15	Sensitive
Sulfadiazine	50	Sensitive
Tetracycline	30	Sensitive
Carbenicillin	100	Sensitive
Gentamicin	30	Sensitive
Kanamycin	30	Sensitive
Cephalothin	30	Sensitive
Methacillin	10	Resistant
Ampicillin	10	Resistant
Chloramphenicol	30	Resistant
Penicillin G	10	Resistant (with morphological effects)

[a]Performed by the FDA disk-diffusion method on Mueller-Hinton agar (Wick et al., 1974).

Effect of Phenylacetic Acid on Az3

Nuti et al. (1972) indicated that phenylacetic acid had marked morphological and biochemical effects on Azotobacter chroococcum. This compound, when added to cultures of penicillin resistant A. chrooccoccum, resulted in elongated, "giant" cells and an increased number of unusually large PHB granules per cell. These affects occurred in a standard nitrogen-free medium equivalent to Mod I.

Az3 cultures were exposed to phenylacetic acid and several closely related compounds. Several other Azotobacter isolates used in this study were also exposed to phenylacetic acid and its analogs. The concentrations recommended by Nuti as well as lesser concentrations were used. The medium used was Mod I. The results of this experiment are summarized in Table 19.

TABLE 19. Effect of Phenylacetic Acid Analogs on Az3

Additive[a]	Grams Wet Weight of Cells at Indicated Concentration[b]			
	100 ppm	10 ppm	1 ppm	0 ppm
Phenylacetic acid	ng	ng	0.8	2.7
Phenyl acetate	ng	ng	0.5	2.5
Phenoxyacetic acid	0.6	0.6	1.3	2.5
Phenoxyethanol	ng	ng	1.3	2.6
Phenol	ng	ng	0.8	2.5

[a]Filter sterilized and added to Mod I medium after autoclaving.

[b]Grams wet weight of cells in 100 ml of Mod I medium after 36 hours. ng = no growth.

At the concentrations recommended by Nuti et al., no growth occurred, while at lesser concentrations, growth was slow and no unusual morphologies were observed.

Final Growth Studies

Final growth studies were carried out in New Brunswick fermentors with ten liters of Mod IV or Mod V medium using Az3 as a PHB producer. These studies used the data from the preliminary growth studies as a basis for obtaining the final fermentation optima.

Aeration

Aeration was varied from zero to 300 cc air/minute/liter of medium using the aeration controls of the New Brunswick fermentor. This study was performed with Mod V medium (containing fixed nitrogen as nitrate). The data from this final aeration study are presented in Figure 22.

Antifoams

Foaming is a problem in aerated fermentations, although not in the shake flasks. Foaming may be severe after 12-18 hours of growth when cell mass increases (and presumably capsular material in solution as well as protein increases). This capsular material is evident on solid media and persists until the cells begin encystment, whereupon the heavy slime layer surrounding the cells gradually disappears. The capsular material may be recovered from spent fermentation broth by mixing the spent broth 1:1 with 95% ethyl alcohol.

Foaming after 12-18 hours of incubation in aerated fermentors was occasionally severe enough to cause a foaming over of the fermentor with

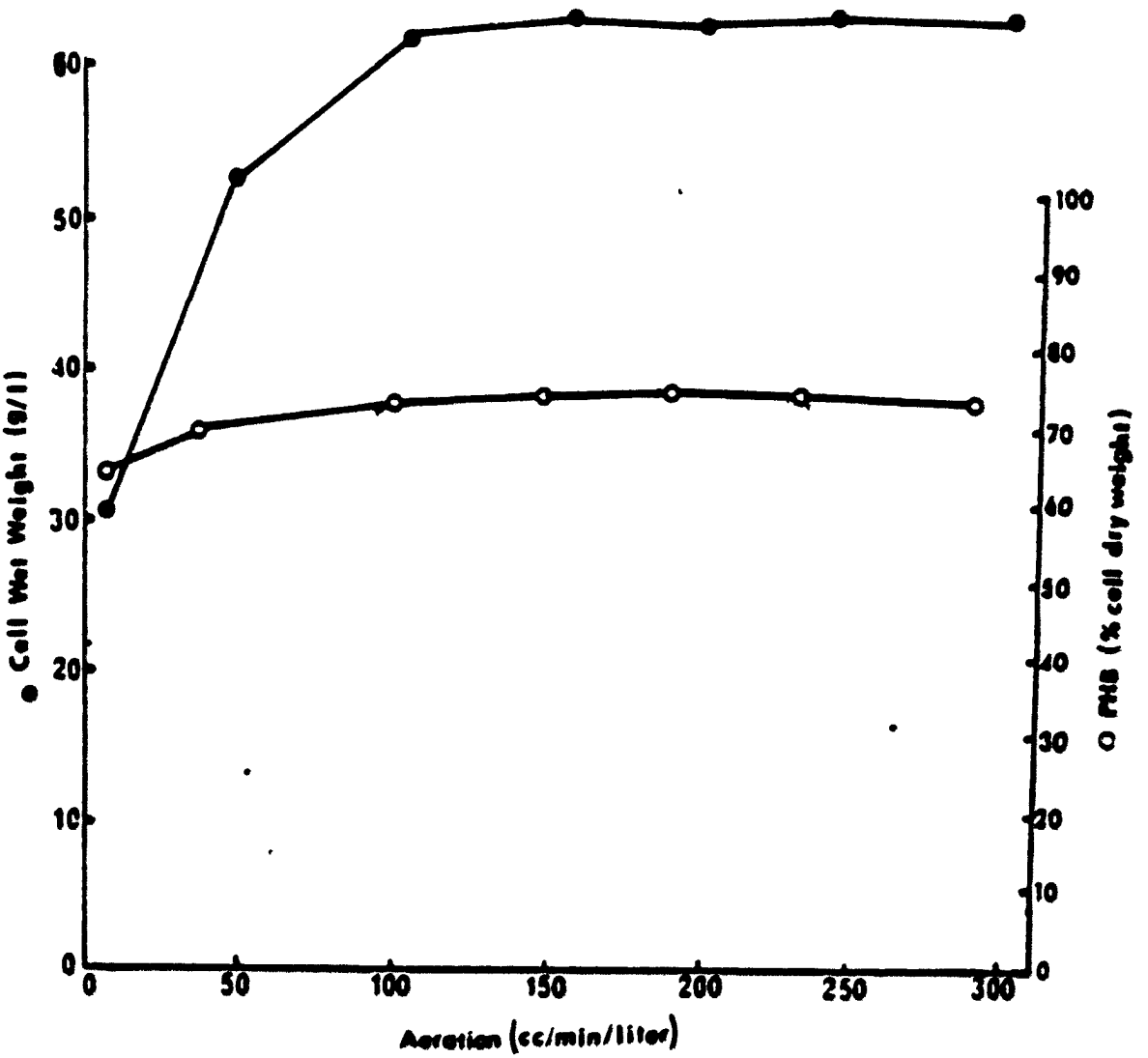


FIGURE 22. Cell Mass and PHB Content of Az3 vs. Aeration Rate

resultant contamination. Silicon based antifoams were successful at controlling this problem when added prior to inoculation, or at any time prior to a foaming over. In order to discover whether less expensive antifoams could be used to control this problem, several commercial oils were tested for their antifoam abilities. These oils were tested in aerated New Brunswick fermentors and in shaken Erlenmeyer flasks. All

oils were successful at controlling foaming in the fermentors, but were generally unsuccessful in flasks. The performance of various antifoams in flasks is summarized in Table 20.

TABLE 20. Antifoam performance in Flasks

Antifoam Type	Recovered Cell Mass[a] (g/l)
Silicon Spray (15 second spray)	31.5
Sigma Silicon Emulsion (0.5 ml/l)	31.7
Peanut Oil (1.0 ml/l)	22.5
Soybean Oil (1.0 ml/l)	23.5
Safflower Oil (1.0 ml/l)	21.8
Standard Deviation =	2.5 g

[a]Grams wet weight in 100 ml of Mod V medium with a 1% inoculum. Incubated at 30° C with shaking for 36 hours. Antifoams were sterilized by autoclaving and added to the sterile medium after cooling.

It should be noted that large quantities of oil had to be added to the fermentors (10 ml per ten liters) in order to control foaming.

Vitamin supplements

Because of the increase in cell yields when molasses was added to fermentation media in preliminary studies, various vitamins were screened for the ability to increase yields of cell mass or PHB. Preliminary screening was performed on solid media with filter paper disks, and vitamins with a zone of increased growth around these disks were selected for further studies in liquid media. The results of these tests are presented in Table 21.

Final growth curves

Growth studies in New Brunswick fermentors using ten liters of the final formulation Mod V medium were performed using the optimal conditions calculated from previous studies. Aeration rates, temperature, carbohydrate, and nitrogen concentrations were rechecked in the large fermentors. The growth of Az3 in the fermentors with fully modified Mod V medium at 33° C with an aeration rate of 100 cc/minute/liter is presented in Figures 23, 24, and 25.

The generation time (G) for the growth of Az3 under these conditions is 3.1 hours (with cell numbers obtained by dilution and viable cell count on nitrogen-free agar). The generation time is reduced to 2.7 hours at higher aeration rates (150 cc/minute/liter) but PHB content is reduced at this aeration rate. The yield of biomass (grams/mole glucose) Y_1 = 62.1 g/M in this fermentation, and the yield of PHB

TABLE 21. Effect of Vitamins on Cell Mass and PHB in Az3

Supplement[a]	Increased growth around disk at 30° C	Cell mass[b] (grams/l wet weight)	PHB (% dry weight)
Thiamine (B_1)	-		
Biotin	+	23.1	68
Nicotinic Acid	-		
Riboflavin (B_2)	+ (?)	23.8	70
Pyridoxal (B_6)	+	22.5	66
Pantothenic Acid	+	24.2	68
Lipoic Acid	-		
Tetrahydrofolic Acid	-		
p-Aminobenzioc Acid	-		
Cyanocobalamin (B_{12})	- (?)	19.5	65
Folic Acid	- (?)	22.2	64
Molasses	+	30.8	67
Control (no supplement)		21.5	65

[a]Cells were grown in Mod III medium without vitamin solution. The medium was solidified with 1.5% Noble Agar (Difco) for disk studies.

[b]Cells grown as above, vitamin solution replaced with 1.0 mg/l of the vitamin indicated. Cells were grown in 100-ml quantities and harvested at 36 hours post-inoculation.

(grams/mole glucose) Y_2 = 44.7 g/M. The yield of PHB is highest at a growth rate slightly lower than maximal (less aeration).

Both the cell mass recovered and the PHB recovered reached a maximum after 36 hours of growth, and did not decline within 48 hours. The

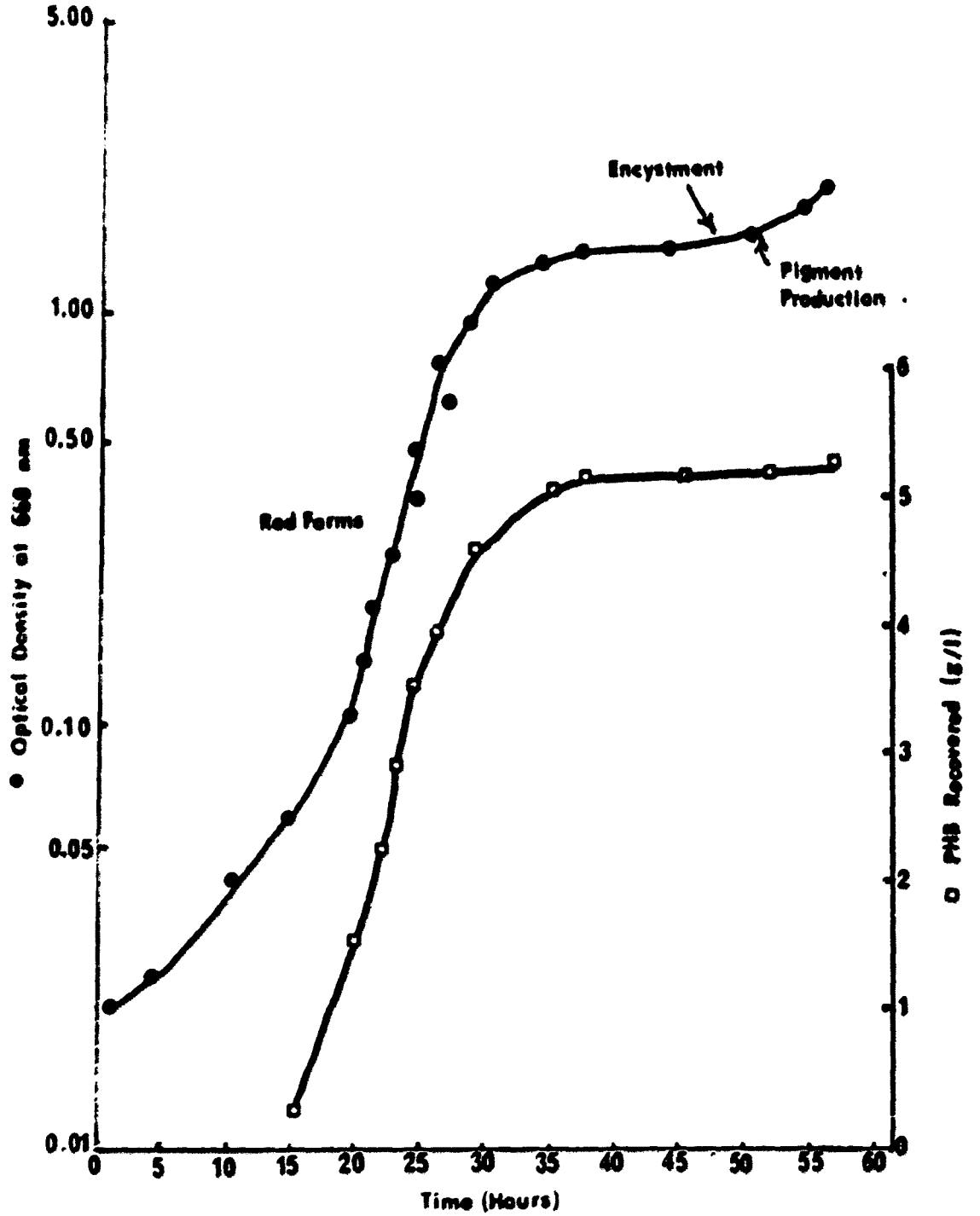


FIGURE 23. Growth of Az3 in Mod V Medium at Optimum Conditions

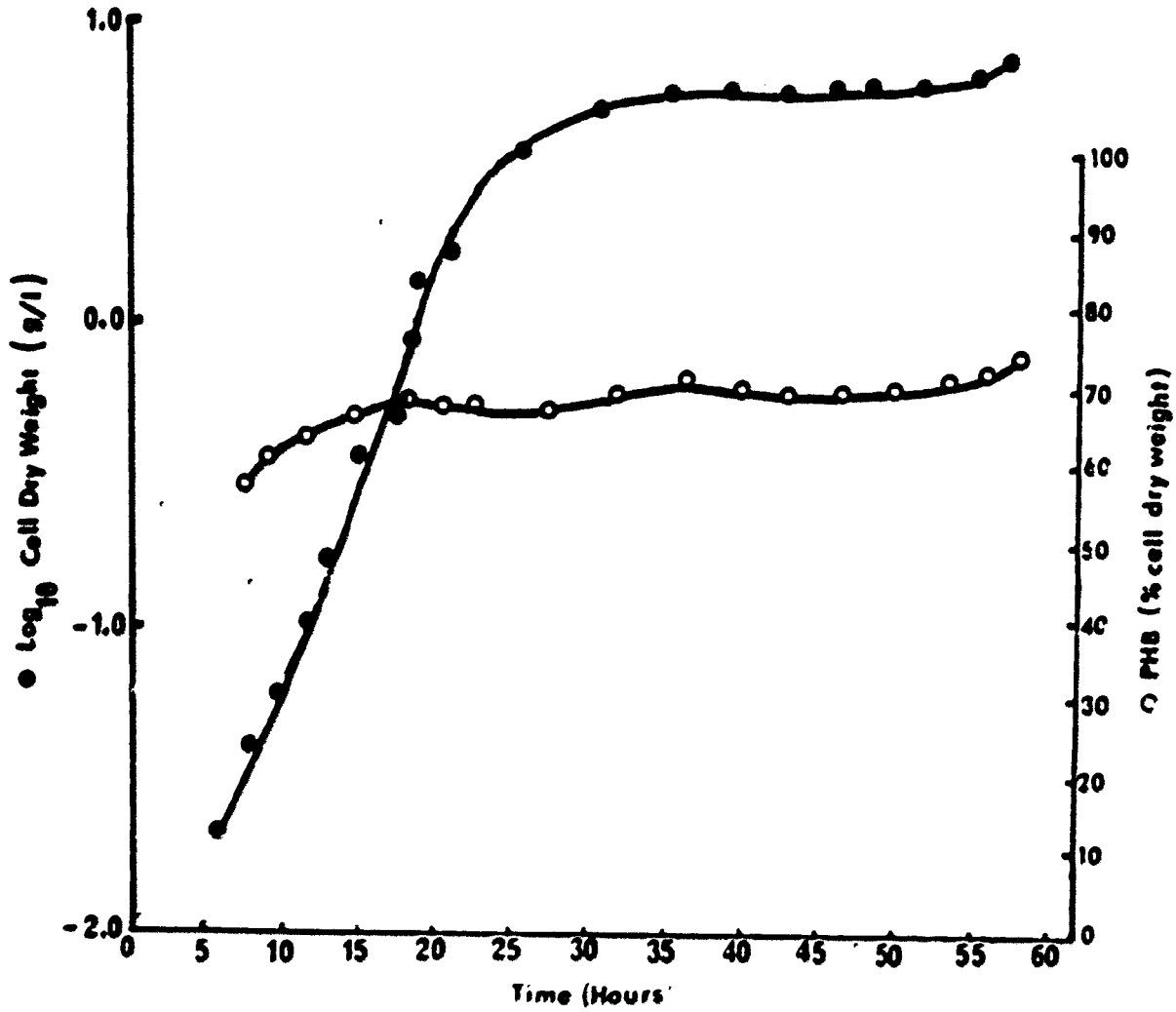


FIGURE 24. Az3 Growth and PHB Content Under Optimum Conditions

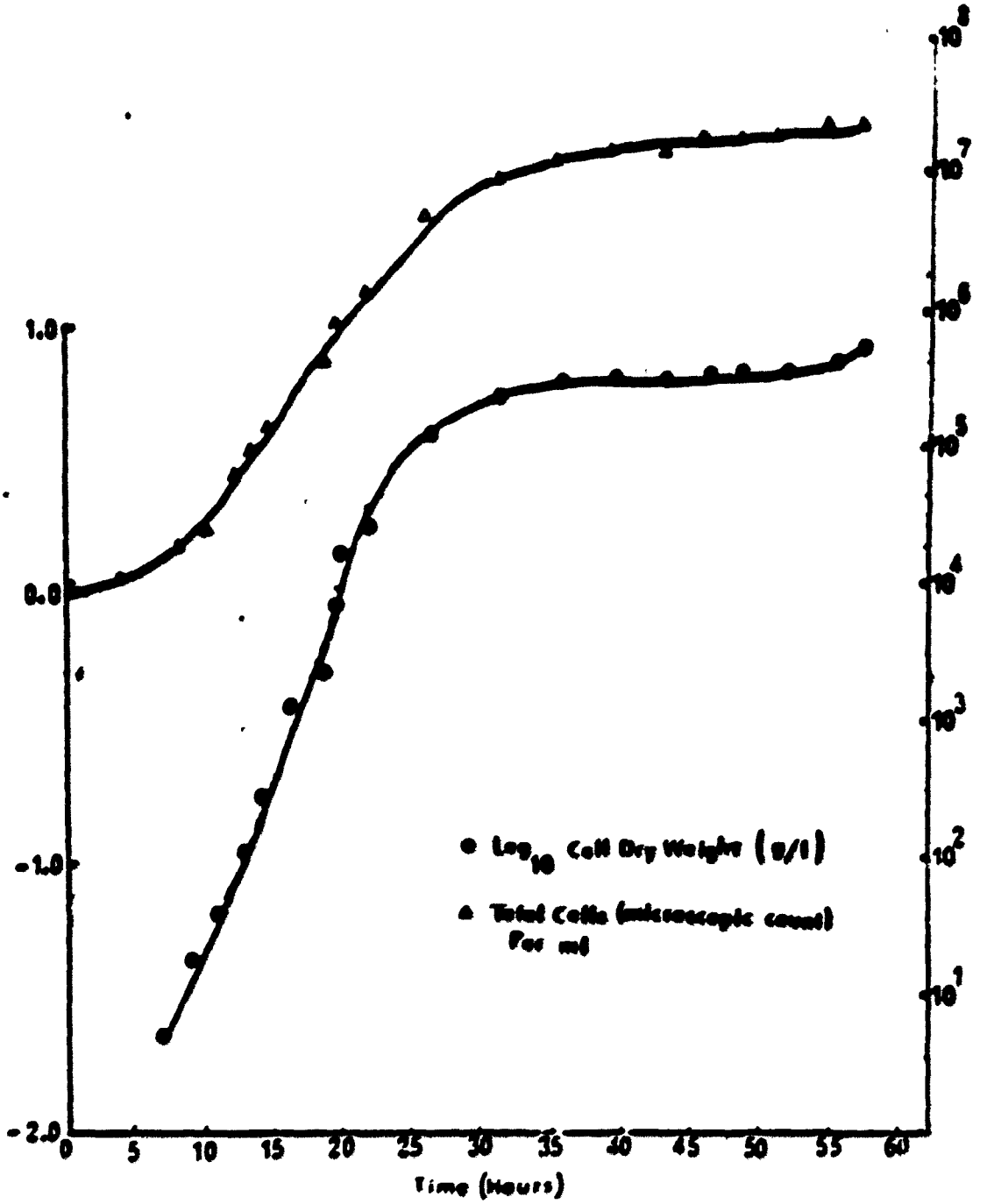


FIGURE 25. Az3 Cell Mass and Cell Numbers Under Optimum Conditions

maximum amount of PHB produced by Az3 under these conditions is 75% cell dry weight.

The amount of PHB per cell is compared to the dry weight of individual cells in Table 22. This is calculated from the weight of PHB recovered from individual samples, the total number of cells detected in the sample, and the weight of the cells recovered in the sample. Characteristic samples from early log phase to stationary phase growth are presented.

The growth of these three slime-reduced mutants of Az3 was investigated under the same conditions. The data for all three mutants were very similar and the growth curve for one such mutant is presented in Figure 26.

The generation time for this mutant is 3.3 hours (obtained as above). The yield of biomass and the yield of PHB per mole of glucose are identical to those reported for Az3.

None of these three mutants demonstrated a requirement for any of the vitamins screened above.

Effect of nitrate on PHB production

To investigate this effect, mutants unable to fix atmospheric dinitirogen were grown in media containing no fixed nitrogen,nitrate nitrogen, amino nitrogen, or ammonium nitrogen. A spontaneous revertant which produces the nitrogenase system even in the presence of reduced

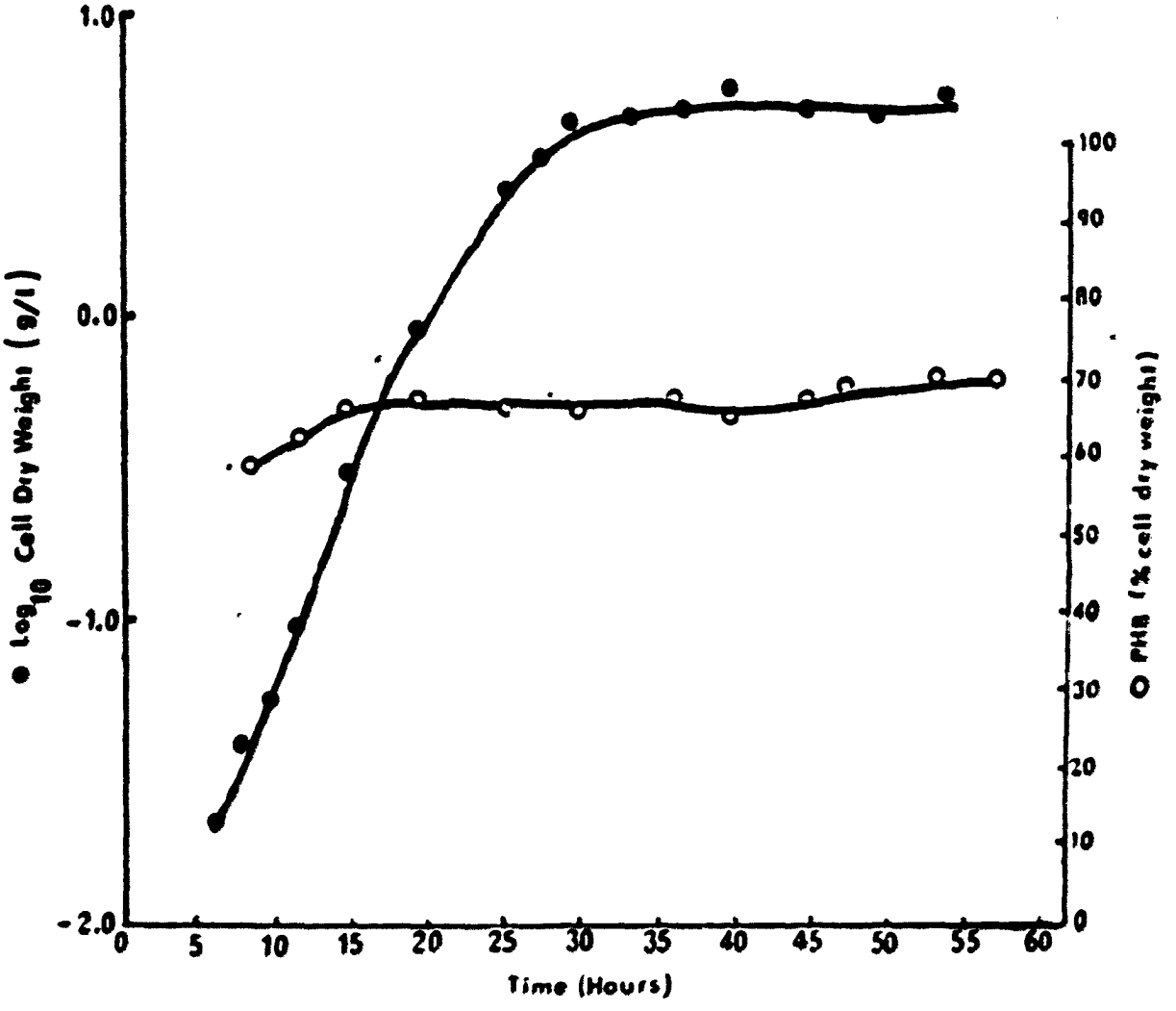


FIGURE 26. Growth of a Slimeless Mutant of Az3

nitrogen sources was also grown under these conditions. These data are presented in Table 23.

Although the nitrogenaseless mutants were able to grow on media containing fixed nitrogen sources such as ammonium chloride, potassium nitrate, or casamino acids, PHB was not produced in significant quantities in any of these cases. Also, the revertant strain, which produces the nitrogenase system even in the presence of a reduced nitrogen source, did not produce significant PHB. This is in direct contrast to the wild strains, which produce PHB when either atmospheric dinitrogen or nitrate nitrogen is supplied, but not when a reduced nitrogen source is supplied.

Optimization of Extraction Methods

Various methods for the extraction of PHB were compared to determine the most efficient method for extracting large quantities of PHB from recovered cells. The thioglycollate extraction method of Nuti et al. (1972) was found to be completely unsatisfactory. Yields of PHB using the thioglycollate extraction method are so low compared to PHB recovery from identical cells using the hypochlorite extraction method that less than 5% of the cell PHB was recovered. This yield is considered so low that it is not significant and further studies on this method were not made. Microscopic inspection of cells extracted by the thioglycollate method revealed that the majority of cells were intact (over 90%). Since this method depends on the dissolution of cells by the thioglycollate (Nuti et al., 1972), it is likely that a failure of the

TABLE 22. Ratio of PHB to Cell Weight During Growth

Cell Number[a] (total count)	Cell Weight[b] (ng/cell)	PHB[c] (ng/cell)	Ratio Weight PHB/Weight Cell
2.71×10^4/ml	0.42	0.30	0.30/0.42 = 0.71
1.26×10^6/ml	0.44	0.31	0.31/0.44 = 0.70
1.16×10^7/ml	0.46	0.33	0.33/0.46 = 0.73

[a] Cell number by microscopic count.

[b] Cell dry weight (dry weight/ml/cell count).

[c] PHB isolated by hypochlorite digestion.

relatively mild thioglycollate reagent to disrupt the cells is responsible for the low recovery level of PHB by this method.

Extraction of PHB was performed by a modification of the original hypochlorite extraction method of Williamson and Wilkinson (1958). The wash and extraction steps were varied to determine the optimum solvent type and quantity for each step. These data are presented in Table 24.

Color in the precipitate remaining after hypochlorite digestion was taken as evidence of incomplete digestion. When such color existed, PHB assay by the acid degradation method invariably detected multiple peaks in the ultraviolet spectrum as opposed to the single broad crotonic acid

TABLE 23. Effect of Nitrogen Source on PHB Production by Nitrogenase Mutants

Nitrogen Source[a]	PHB Production as Percent Cell Dry Weight in Strain[b]							
	1	2	3	4	5	6	7	Az3
None	ng	ng	ng	ng	ng	ng	22.7	72.1
KNO_3 (0.01%)	0	0	0	0	0	0	10.1	73.0
$(NH_4)_2SO_4$ (0.05%)	0	0	0	0	0	0	0	0
NH_4Cl (0.01%)	0	0	0	0	0	0	0	0
Casamino Acids (0.5%)	0	0	0	0	0	0	0	0
Yeast Extract (0.5%)	0	0	0	0	0	0	0	0

[a]Mod IV medium was used as a basal medium for this study. Cultures were grown in 1 liter quantities in Fernbach flasks at 30° C with shaking, and harvested at 36 hours.

[b]Strain 1 = Az3 nifa, strain 2 = Az3 nifb, strain 3 = Az3 nifc, strain 4 = A. vinelandii nif⁻, strain 5 = A. vinelandii n1, strain 6 = A. vinelandii n2, strain 7 = A. vinelandii revertant. ng = no growth. 0 = cell growth but no PHB production.

TABLE 24. Determination of Extraction Optima

Variation in Basic Scheme (see Table 23)	% Yield[a]
Basic	100
5 H_2O washes in step 1	93
No H_2O washes in step 1	112 (color)
Extract in 10:1 1N NaOH	--- (no PHB)
15:1 hypochlorite:cells	93
12:1 hypochlorite:cells	91
10:1 hypochlorite:cells	95
6:1 hypochlorite:cells	101 (color)
4:1 hypochlorite:cells	106 (color)
3:1 hypochlorite:cells	120 (color)
Wash 10X in tap water, step 3	92
Wash 15X in tap water, step 3	90
Wash 4X in solvents, step 4	94
No ETOH wash, step 4	95 (inter.)
No acetone wash, step 4	110 (inter.)
Eliminate step 5	105 (inter.)
Step 5, double wash	101
Eliminate step 6	109 (inter.)
Dichloromethane extraction, not boiled	81
Chloroform extraction, not boiled	53
Dichloromethane extraction, boiled	83
Dry polymer without ETOH precipitation	104

[a] PHB as a percentage of the unmodified scheme, cells grown in Mod III medium and pooled prior to assay. Cells were harvested at 36 hours. Color = color noted upon visual inspection of the cell digest. Normal color at this point is white. Inter = interference in assay, a UV spectrum other than that of crotonic acid observed.

peak expected. "Interference in assay" indicates the existence of multiple peaks in the UV spectrum, indicating contamination.

The final, optimized PHB extraction method is outlined in Table 25. The step numbers are the same as the steps varied in the optimization procedure.

Although hypochlorite is expended by this method and cannot be recovered, the acetone, ether, ethanol, chloroform, and dichloromethane can be recovered by distillation. Recovery of these reagents has been performed on a trial basis, and PHB extraction using recovered reagents does not appear to be less efficient than recovery using fresh reagents. No attempt was made to recover polyethanol carbonate.

The Effect of Nitrate on Encystment in *Azotobacter*

The genus *Azotobacter* is known to possess a life cycle which includes the formation of resistant forms classified as cysts. These metabolically dormant forms are formed by a process of cell differentiation and contain a definite intine and exine as well as novel carbohydrates. This process has been reviewed by Sadoff (1975).

During the growth studies performed on Mod II medium, it was noted that *Azotobacter* cells formed definite cysts in this medium. According to Sadoff, nitrogen starvation is necessary for the encystment cycle to commence. Because of this contradiction between Sadoff's criteria for

TABLE 25. Extraction of PHB

1. Collect cells by centrifugation, wash once in 8% (v/v) aqueous acetic acid, twice in nonsterile tap water.
2. Place cells in hypochlorite reagent (8:1 hypochlorite:cells).[a] Digest 12 hours at room temperature with stirring.[b]
3. Wash five times with tap water.
4. Wash twice each in 95% ethanol and acetone.
5. Wash one in acetone:ether (1:1).
6. Soak in acetone:ether (1:1) overnight.
7. Extract PHB in boiling 1% ethylene chloride in chloroform.[c]
8. Separate the viscous mixture by centrifugation or in a separatory funnel.
9. Precipitate PHB from the chloroform by mixing 2:1 chloroform-PHB: 95% ethanol or by drying overnight in a vacuum desiccator.
10. Assay precipitated PHB.

[a]Hypochlorite solution is commercial bleach.

[b]The solution is stirred in an Erlenmeyer flask on a magnetic stirrer at the highest speed possible with the liquid volume/stirrer combination used.

[c]Extraction is best performed in a separatory funnel.

encystment and the behavior of Azotobacter in Mod II medium, which was supplemented with nitrate, a series of studies were performed to investigate the relationship of nitrogen source type to the occurrence of encystment. These data are presented in Table 26.

TABLE 26. Effect of Nitrogen on Encystment

Medium[a]	Strain[b]	
	Az3	Az19
Mod IV without vitamins	abortive	abortive
Mod V without vitamins	abortive	abortive
Mod V with NH_4Cl (1g/l)	no cysts	no cysts
Mod V with 1% glucose	encysted	encysted
Mod IV with 1% glucose	encysted	encysted
Mod V with NH_4Cl (1g/l) and 1% glucose	no cysts	no cysts
Mod IV + n-butanol[c]	abortive	abortive
Mod V + n-butanol	abortive	abortive
Mod II	encysted	encysted
Mod II + n-butanol	encysted	encysted
Mod II + 1g/l KNO_3	encysted	encysted
Mod II + NH_4Cl (1g/l)	no cysts	no cysts

[a] Mod II is nitrogen-free unless otherwise indicated; Mod IV is nitrogen-free unless otherwise indicated, Mod V contains 1.0 g/l KNO_3 unless otherwise indicated. When a specific nitrogen source is indicated, it is in place of the normal nitrogen content of that medium. When a specific carbohydrate content is indicated, it is in place of the normal carbohydrate content of that medium.

[b] Abortive = abortive encystment, encysted = complete encystment. Samples were taken at 5 hour intervals for 150 hours.

[c] N-butanol was added in 0.2% quantities after autoclaving.

DISCUSSION

Staining and Assay with Nile Blue Sulfate

Although the amount of PHB in various samples can be estimated by measuring the fluorescence detected after NBS staining, this method was considerably less sensitive than the assay of PHB by hypochlorite extraction and acid degradation. For this reason, NBS staining was used as a rapid qualitative assay of PHB, but was not used as a quantitative assay.

Assay of PHB with NBS fluorimetry, as shown in Figure 13, was also time-sensitive. Since observed fluorescence declined sharply as more time elapsed between the addition of NBS to cells and fluorimetry readings, this method was not suited to critical quantitative work. Even time delays of less than one minute between NBS addition and fluorimetry resulted in declines in the detected fluorescence. It is also apparent that the detected fluorescence remained constant, or rose, even as the amount of PHB recovered by hypochlorite degradation of the cells declined after 90 hours of growth. This may be due to the process of encystment or abortive encystment which occurs at this time. No fluorescent structures other than PHB granules were noted in these cells. Stained, fluorescent granules were readily seen within cysts and abortive cysts at this time, but the amount of PHB recovered by hypochlorite degradation declined. Although fluorescent granules were still seen in encysted cells, and seemed to occupy the majority of the cyst, the cyst

itself was smaller than a vegetative cell, which may account for the tendency to overestimate the amount of PHB in encysted cells when visual estimation or fluorimetric estimation was used.

NBS was superior to Sudan Black B for staining PHB granules within bacterial cells. More PHB granules were stained and there was less danger of over-decolorization. The ease of visually detecting orange fluorescence against a dark field makes the detection of scattered PHB granules in only a few cells easier with NBS staining as compared to Sudan Black B staining. A disadvantage of NBS staining for the detection of PHB granules was the need for an epifluorescence or fluorescence microscope whereas a normal brightfield microscope is all that is needed for Sudan Black B staining.

The quantitative assay of PHB by visual estimation of the amount of fluorescent PHB granules in stained preparations was not as sensitive as determination of the weight of isolated PHB. Several strains which were rated as high PHB producers by visual estimation of PHB granules in Table 9 had less PHB as a percentage of cell dry weight than strains which visually appeared to have less PHB. This is apparent from a comparison of the data in Tables 9 and 10.

NBS staining was also quite successful as a qualitative assay for the presence of PHB within colonies when colonies replicated onto glass-fiber filters. This replica-staining technique allowed the screening of large numbers of isolates for the presence of PHB. The replica-staining technique should never be used to estimate the relative amount

of PHB in a given colony, as it was impossible to reliably detect small differences in the intensity of fluorescence.

It can be concluded that NBS is a more reliable stain for PHB than is Sudan Black B. Nile Blue Sulfate is less easily removed from the cells by a decolorization step, and unstained granules are seldom observed microscopically. Unstained granules are frequently observed with Sudan Black B. Further, the staining of colony replicas with NBS allows the rapid screening of large numbers of isolates for the presence of PHB.

Preliminary Growth Studies

Growth Curves

A growth curve for Az3, a strain which produces a high level of PHB, is presented in Figure 14. In a nitrogen-free medium with glucose as a carbon source, Az3 entered log phase growth within 6 hours of inoculation when encysted cells were used as an inoculum (based on optical density). Log phase ended about 30 to 36 hours after inoculation, and optical density then remained steady until about 50 hours after inoculation when the cells began to form cysts. These cysts were visible as distinct spheroid forms (as opposed to the fat rod forms noted during log phase). At this point, a soluble brown pigment was produced and the entire medium eventually turned brown or black. This pigment production caused a rise in optical density but no rise in the total number of cells was observed microscopically.

PHB content as a percentage of cell dry weight increased slightly after the inoculum entered log-phase growth, but declined when cyst formation occurred. Thus, cyst formation is concurrent with pigment formation, the appearance of spheroid cell forms, and a decline in PHB levels. These data are in agreement with the observations of Sadoff (1975), who believed that PHB was metabolized during cyst formation.

As is apparent from the data presented in Table 12 and Figure 16, Az3 produced the highest levels of PHB (as a percent of cell dry weight) and the highest optical densities during growth when glucose, sucrose, fructose, or soluble starch were supplied as carbon sources. In Figure 16, it may be noted that log phase growth ended at 34-44 hours except when starch was used as the carbon source, where no clear end to log phase was evident. The probable end to log phase in starch-based media was around 55 hours under these conditions (based on samples taken as late as 190 hours after inoculation). It is clear that cell mass was highest when glucose was used as a carbon source. Pigment production was also noticeable in the later stages of the fermentation. Glucose was chosen as a carbon source for use in further studies because of its successes in this preliminary experiment.

When the amount of glucose in the medium was varied (Figure 17), concentrations of glucose above 2.0% (w/v) did not result in increased cell mass. PHB levels remained fairly constant as a percentage of cell dry weight in samples taken 36 hours after inoculation (at or near the end of log phase). When the initial concentration of glucose in the

medium was below 2.3%, the PHB content of the recovered cells decreased in samples taken at 60 hours. This indicated that there was a decline in the PHB concentration within the cells with the onset of stationary phase. This conclusion is supported and amplified by the data in Figure 15. PHB concentration, in a medium containing 1% glucose, initially reached levels comparable to those obtained with higher glucose concentrations, but then declined with the onset of stationary phase. This decline in PHB levels corresponded with the appearance of cysts in the medium. This phenomenon is similar to that noted by Sadoff (1975) in Azotobacter vinelandii.

This decline in PHB concentration with cell encystment was not apparent at higher glucose concentrations (i.e. 2.3%). It may be concluded that when excess glucose was present in the medium, cellular PHB levels did not decline in the later stages of the fermentation. When initial glucose levels in the medium were 2.3% or above, Az3 cells did not encyst. The PHB concentration declined, however, if the cells encysted (for example, at lower glucose levels). This may indicate that PHB is used to provide energy and/or carbon for the process of encystment. It must be remembered that PHB was still present in the cysts, at levels of 20% of the cyst dry weight in 60-hour-old cultures grown in 1% glucose.

Because of the results of this experiment, 2.3% glucose was used as a standard carbohydrate content for growth media. Above this concentration, total cell mass did not increase, while PHB remained a

constant (or decreased) fraction of the cell dry weight and did not decrease in stationary phase. The fact that PHB did not decline rapidly in stationary phase when a 2.3% glucose concentration was used makes the harvesting of cells an easier task because the harvesting time would not be critical.

Nitrogen sources

Cell mass increased when nitrogen was supplied in the medium and the cells no longer must fix atmospheric dinitrogen. The PHB content was high (comparable to that found in previous experiments to determine optimism glucose concentration) when no inorganic nitrogen was supplied or with an oxidized nitrogen source (i.e. potassium nitrate). The PHB content decreased dramatically when a reduced nitrogen source (i.e. ammonium chloride) was supplied. When a reduced nitrogen source was supplied, very few PHB granules were seen with NBS staining.

Potassium nitrate is utilized by the cells because the nitrogen-fixation mutants obtained by UV irradiation grew well on Mod V medium, which contained potassium nitrate. These mutants did not grow on nitrogen-free agar. It is also known that members of the genus Azotobacter are capable of utilizing nitrate nitrogen (Brill, 1974). Thus,it would appear that these organisms did not continue to fix atmospheric dinitrogen when nitrate nitrogen was supplied, although the Az3 will type may produce nonfunctional nitrogenase enzymes or other nitrogenase-system components when nitrate is supplied.

The PHB content was relatively constant when 0.01 to 0.15% potassium nitrate was added to the medium. However, total cell mass, and thus the total amount of PHB recovered upon cell harvest, increased until 0.01% potassium nitrate was reached. The accumulation of PHB when nitrate was present in the medium seemingly contradicts the belief of many researchers (Senior and Dawes, 1971a, Senior et al., 1972, Peterson and Hsu, 1977, Ward et al., 1977) that nitrogen-free media coupled with the fixation of atmospheric dinitrogen is necessary for PHB production in Azotobacter. The repression of PHB synthesis when fixed nitrogen sources are present in the growth medium is found only where reduced nitrogen sources are present, as indeed they are in most commercial complex media.

Because of the results of this experiment, 0.01% potassium nitrate was incorporated into Mod V medium.

Medium constituents

Iron content The data on the effects of iron on Az3 are presented in Table 15. PHB content as a percentage of cell dry weight was unaffected by iron content. Cell mass was affected (declined) below 0.0005 g/l $FeCl_3$. $FeSO_4$ was also added to the medium to determine if a different iron source affected PHB level or cell mass. It ia apparent that PHB can utilize iron in either form, and $FeSO_4$ has no ability to increase yields. Indeed, slightly lower cell yields are noticeable when $FeSO_4$ was used. It is

probable that iron is present in other media constituents and thus is present even in the "iron-free" controls. These iron requirements and their levels are fairly standard in media for Azotobacter, and these results are not surprising.

Phosphate content Recovered cell mass was constant above 0.25 g/l added K_2HPO_4, and PHB content (as percent cell dry weight) did not decline until concentrations of less than 0.17 g/l were reached. Cell mass also declined rapidly at levels below 0.17 g/l K_2HPO_4. 1.0 g/l K_2HPO_4 is used in Mod V medium which obviously contains excess phosphate. This level of K_2HPO_4 is necessary for its buffering capacity. Az3 is sensitive to acid conditions and a 2.3% glucose content puts demands on the buffer capacity of standard nitrogen-free media that contain a low K_2HPO_4 level. Thus, as long as Mod V medium is in use, these data are of academic interest only.

Calcium content Calcium carbonate is used in high amounts for its its acid-neutralizing capacity. Mod V medium and all other nitrogen-free media used in this work contained excess calcium, which was visible as undissolved calcium carbonate in the medium. It is apparent from the data in Table 15 that the 10.0 g/l calcium carbonate often used in nitrogen-free media for the growth of Azotobacter is in excess of the required

amount. Indeed, a calcium carbonate level of 0.5 g/l, as used in Mod V medium, is sufficient. This is less than a tenth of the concentration of calcium carbonate normally found in Norris' nitrogen-free medium, which is the basis for most media used for the growth of the Azotobacteraceae.

Trace minerals As can be seen from the data in Table 16, PHB content as a percentage of cell dry weight was unaffected by any of the supplements used, including molasses. However, the recovered cell mass and thus the total amount of PHB recovered was increased in some instances. Total cell mass was increased when molasses, ashed molasses, trace mineral solution, magnesium, manganese, and molybdenum were used. In some cases the increases in cell mass were very small, and were at the edge of statistical significance.

As a result of this study, the concentrations of magnesium and molybdenum in Mod III medium were modified to 0.2 g/l $MgSO_4 \cdot 7H_2O$ and in 1.0 mg/l quantities.

As indicated in Table 17, sufficient quantities of these minerals are probably supplied when an impure source of water was used. As all possible combinations of mineral which showed no immediate growth effects were not tested, the possibility still remains that one of the minerals not showing a growth-enhancing effect may do so when other limiting factors are eliminated.

pH effects

The content of PHB in Az3 cells as a percentage of dry weight was not seriously affected by a drop in pH until long after cell growth was in decline. pH 7.4 was an acceptable pH level for the growth of cells and production of PHB by Az3. Below pH 4.5, no growth could be detected. Growth below pH 5.8 was slow. The accumulation of PHB as % cell dry weight was adversly affected below pH 5.8.

It is apparent that PHB production within the cells was less sensitive to acidic conditions than was the total cell mass. The cause of this phenomenon is not known, but it may well be that the metabolic pathways and enzymes responsible for PHB production are less sensitive to low pH than are other enzymes or pathways necessary for cell growth.

Temperature effects

As can be seen from Figure 20, Az3 grew well at temperatures ranging from room temperature to 37° C. The temperature optimum for both growth and PHB production would appear to be 33° C. This is a higher temperature than the optimum temperature of around 30° C usually reported for Azotobacter (Johnstone, 1974). Growth at a higher temperature is desirable, not only because of theoretical increases in metabolic rate as temperature increases, but also because higher growth temperatures may eliminate some of the cooling expense for large fermentations.

Due to the results of this study, a fermentation temperature of 33° C was adopted for the production of PHB with Az3. These data would also indicate that many of the published temperature optima for the Azotobacteraceae may not be as universal as previously thought.

Aeration

Since Azotobacter is a strict aerobe with an oxygen-sensitive nitrogenase system, it has been theorized that a high respiration rate is used by the organism to lower the oxygen concentration in the immediate area of the nitrogenase system. In order to maintain a high respiration rate, the organisms must maintain a supply of reduced coenzyme (NADH) which can be recyled by the cytochrome system. Synthesis of PHB is a process competing with nitrogen fixation as PHB synthesis also requires reduced coenzyme. Thus, it is interesting that PHB accumulation has traditionally been associated with nitrogen fixation (Dawes, 1975).

It is also possible that the nitrogenase is protected by external slime or by the large size of Azotobacter cells. Large size would mean a lower surface area (for oxygen entry into the cell) for a given cell volume. The association of membranes with enzymes of the nitrogenase system may also serve as a physical barrier to protect the oxygen-sensitive components. This membrane/nitrogenase relationship has been discussed by Oppenheim and Marcus (1970). It is possible that Azotobacter uses a combination of cell size, slime, clumping, membranes, and respiration rate

to protect its nitrogenase system from oxygen. In this context, it is helpful to recall that slime disappeared from encysted Azotobacter colonies on solid nitrogen-free media (i.e. Mod I). Azotobacter nitrogen fixation has been reported to cease with the onset of encystment (Sadoff, 1975). Thus, if the slime has an oxygen-limiting effect for nitrogen-fixing cells, it would no longer be needed for this purpose after encystment began, which may account for its disappearance at this time.

PHB accumulation declined at relatively high aeration rates when nitrate supplied (Figure 21), but did not decline at aeration rates of over 150 cc/min/liter when nitrate supplied (Figure 22). This decline in PHB levels at the higher aeration rates (in this case over 150 cc/min/liter) has been noted by other researchers (Dawes, 1975), but the moderation of this effect by nitrate has not been reported prior to this study. This moderation in PHB decline at other than minimal aeration rates obviously has significance in understanding the rationale behind the production of PHB in the Azotobacteraceae. PHB is not formed merely as a consequence of nitrogen-fixation, because it has already been demonstrated in this study that PHB accumulates to high levels with nitrate in the medium. With nitrate present, PHB was also formed at high aeration levels, whereas it was not formed in large amounts when nitrate was not supplied at these aeration levels.

An aeration rate of 150 cc/minute/liter was apparently sufficient for growth and PHB production by Az3.

Antibiotic and phenylacetic acid effects

Antibiotic sensitivity was originally performed to determine whether an antibiotic could remove contaminating organisms (usually pseudomonads), without affecting the growth of Az3. Ampicillin (10 mg/l), was added to some fermentations to help prevent contamination, and no effect on cell mass or PHB content was noted. No antibiotic tested was noticeably helpful in removing pseudomonad contaminants embedded in clumped Az3.

The antibiotic sensitivities in Table 18 are interesting when compared with the observations of Nuti et al. (1972). Nuti at al. reported that phenylacetic acid added in 100 ppm quantities to Azotobacter cultures in a simple nitrogen-free medium (equivalent to Mod I), resulted in "giant" ribbon-shaped cells with an increased number of PHB granules per cell. These Azotobacter cultures were reported to be penicillin-resistant. The morphological effects described by Nuti et al. are similar to those noted when penicillin was added to Az3 cultures during antibiotic sensitivity tests.

Nuti et al. believed that the morphological effects they noted were related to the production of penicillinase by their organism. This relationship was not further explained by Nuti et al.

It is apparent from the data in Table 19 that phenylacetic acid did not result in morphological changes in Az3 when added in 100 ppm concentration. Indeed, no growth occurred when 100 ppm phenylacetic acid was supplied. The growth which occurs at lower concentrations of phenylacetic

acid did not display morphological effects such as those described by Nuti et al. When various analogs of phenylacetic acid were used, the data supported the conclusion that phenylacetic acid and its analogs produced no morphological effects on Az3 similar to those described by Nuti et al. (1972). The addition of phenylacetic acid or its analogs to nitrogen-free media to increase cell mass or PHB levels per cell was not a valid method with the cultures used in this study. Whether these results could be confirmed with the cultures used by Nuti et al. is not known, because we were unable to obtain these strains.

Final Growth Studies

Antifoams

Foaming was a problem in aerated fermentations, especially in the later stages of the fermentation. Occasionally, cultures in large fermentors foamed so severely that the fermentation had to be halted. Antifoams were added to control this problem.

Although the silicon antifoams performed well and did not affect cell mass or PHB yields, the oil antifoams did depress cell yields (see Table 20). This depression of cell mass (which was not noted in aerated fermentors) may be caused by the detrimental effect of the oil layers on aeration in flask-grown cultures. The aerated fermentors would not be as affected by this phenomenon, because air is introduced from spargers in the bottom

of the fermentor, and aeration is not as dependent on surface diffusion. Surface diffusion of air would be adversely affected by an oil layer on the surface of the broth. Oils were not as reliable in controlling foaming in fermentors as were the silicon antifoams. Oil-protected fermentations occasionally foamed severely, but seldom enough to require abandoning the fermentation.

The slight increase in cell mass noted with soybean oil, as opposed to peanut or safflower oil, may be due to the presence of protein or other impurities in the oil.

Vitamin supplements

Of the vitamins tested, pantothenic acid, biotin, and riboflavin increased growth on solid or liquid media. The results for pyridoxal were less clear-cut, but some increase in cell mass was noted. Riboflavin may also have an effect. Increasing the concentrations of these vitamins to 2.5mg/liter did not result in any further increase in observed cell mass. The amount of PHB accumulated by the cells did not appear to be greatly affected by these supplements.

It should be noted that the producer strain (Az3) did not have an absolute requirement for any of these vitamins. Therefore, the increase in cell mass when certain vitamins were supplied was probably because of increased cellular efficiency when these compounds were supplied to the cell, eliminating the necessity for the cell to expend energy or precursors

to synthesize them.

Not all possible combinations of vitamins were tested, leaving the possibility that a vitamin not limiting in the original screening might become limiting when other vitamins were added. However, because Az3 was able to synthesize all the vitamins it needed for growth, as demonstrated by the fact that Az3 grew well on mineral-salts media without vitamins, the possibility of a vitamins severely limiting growth would seem remote.

Growth curves

As is evident from Figures 23 and 24, PHB levels did not decline after the beginning of stationary phase, and did not decline at any time up to 50 hours after inoculation. Maximum levels of both cell mass and PHB recovered were reached after 36 hours of growth. The PHB levels of 72-75% cell dry weight obtained in these studies were as high or higher than previously reported levels of PHB in Azotobacter. Previously, Akita et al. (1976) had reported 72% cell dry weight PHB in Azotobacter vinelandii, but with lower total cell mass per liter.

As noted in Table 23, levels of PHB were near 30 nanograms/cell, except in stationary phase where 33 nanograms of PHB per cell were found. Cell mass also increased to 46 nanograms per cell (dry weight) in stationary phase. This may be due to the process of abortive encystment which occurred at this time. As the cells encyst, an intine and exine are formed and the cells become spheroidal. At the same time, cell numbers increase

only slightly (perhaps due to the fact that fewer double cells are seen after encystment or abortive encystment). The total cell mass recovered remains fairly constant as abortive encystment begins. Thus, if cell dry weight increases as abortive encystment occurs, and the PHB level as a percentage of cell dry weight remains fairly constant, PHB mass must increase slightly at this time. This is indeed the case, as Table 22 demonstrates.

Therefore, PHB was not being used by the cell in the process of abortive encystment, and may even accumulate to a greater extent.

It is evident, from the constant nature of the ratios of PHB per cell to cell dry weight as well as from the fairly constant nature of the dry weight of individual cells, that no great loss of cell mass occurred during this time to alter the measurment of PHB as percent cell dry weight.

Excess glucose is present in Mod V medium, so the cells do not fully encyst. Further, this excess glucose (usually about 0.3%) may be available for the process of abortive encystment, eliminating the need to metabolize PHB for this process.

A reduced slime mutant of Az3 was also obtained, and it is evident that the reduction in slime production did not cause this strain to be a less efficient producer of PHB than the parent strain. Apparently, the biochemical pathway affected by the slime-reducing mutation was not connected with PHB production. Further, loss of slime-producing capacity did not seem to affect the growth of the organism in a significant fashion.

Effect of nitrate on PHB production

Senior and Dawes (1971a,b) concluded that PHB was produced by *Azotobacter* as an electron sink in order to maintain a high metabolic rate. The high metabolic rate was needed to help protect the nitrogenase system against oxygen. A high metabolic rate, particularly in oxygen-limited cells, would result in an accumulation of reduced coenzyme (NAD(P)H). An oxygen limitation might well result from the need of the cells to protect the nitrogenase, and might result from high respiration, cell clumping, or slime around the cell, as previously discussed. With such an oxygen limitation, the reoxidation of NADPH and NADH by PHB synthesis would allow continued glucose metabolism. Nitrogen fixation would also be such an electron sink. However, it is possible that nitrogen fixation would be limited by the availability of ATP, or would proceed at too slow a rate to reoxidize enough coenzyme to maintain a high metabolic rate.

If PHB is accumulated in nitrogen-free media only, as widely believed, a necessity to protect the nitrogenase system at the same time as PHB was accumulated would exist. Under these conditions, PHB might be expected to compete with the nitrogenase system for reduced coenzyme. It has been noted by Dawes (1975), Ward et al. (1977), and many others, that PHB is not produced in media containing a fixed (reduced) nitrogen source. The data presented in this study make it evident that PHB is produced by *Azotobacter* in the presence of nitrate nitrogen, and that aeration, which has been reported to inhibit PHB production (Senior and Dawes, 1971a), does not inhibit PHB production when nitrate is present in the medium.

Yet the production of PHB in these organsims does not seem to be connected to the nitrogenase system in some way, as the nitrogenase mutants were notably unsuccessful in producing PHB regardless of their nitrogen source. It may be possible, of course, that these mutants contain multiple mutations, some of which affect the PHB polymerase system or other enzymes related to the anabolism of PHB. This is a distinct possibility, due to the large genome (about ten times that of Escherichia coli), with Azotobacter having little more biochemical diversity than E. coli, it is possible that Azotobacter containe many copies of various genes, making phenotypic expression of a mutational event difficult (Sadoff, 1975).

It would appear that the inclusion of nitrate allows growth of Az3 without a decline in PHB levels, although such a decline would have been expected based on a survey of the literature. Further, the inclusion of nitrate largely reduces the decline in PHB levels in aerated cultures that had been previously reported (Senior and Dawes, 1971a). However, unless a nitrogenase system is present, Az3 and other azotobacters apparently do not accumulate PHB. This indicates a link between PHB metabolism and the nitrogenase system in this genus, although the two characteristics are not always found together. It is possible that in the presence of nitrate, a high metabolic rate is maintained by the cells, producing reduced coenzyme in excess of the needs of nitrate reductase. In this case, PHB production might serve as an electron sink to regenerate oxidized coenzyme. Without the nitrogenase system, it may be that the need to limit the presence of oxygen in order to protect nitrogenase is no longer present.

The presence of nitrate may not eliminate efforts by the organism to limit oxygen concentration.

A further possibility is that PHB is produced in connection with the process of encystment, since neither PHB production nor encystment occur on media containing sources of reduced nitrogen. Interestingly, the nitrogenase mutants formed no obvious cysts on any medium.

Effects of nitrate on encystment

Encystment occurred when a basic mineral medium (Mod II) containing either nitrate nitrogen or no added nitrogen was used. Mod II contains 1% glucose. No cysts formed when a reduced nitrogen source was added to Mod II. Encystment also occurred in Mod IV and Mod V media containing 1% glucose. Media containing higher glucose concentrations (2.3%) supported only abortive encystment when nitrate was present, and no encystment when reduced nitrogen was added.

Abortive encystment is characterized by an incomplete exine and larger cells which are not completely spheroid.

Thus, Sadoff's criteria for encystment must be modified to state that an absence of reduced nitrogen is necessary for encystment, not that all nitrogen must be removed from the medium. It is also interesting to note that cells without PHB (ammonium-grown or nitrogenase mutants) do not encyst. Since the conditions for encystment seem to parallel those for PHB accumulation, with the exception of glucose concentration, it is

possible that PHB stored in the pre-cyst cell is necessary for the process of encystment.

The addition of n-butanol, an inducer of encystment, did not produce cysts when glucose was present in 2.3% (w/v) quantities in the medium prior to inoculation.

The number of cells in a culture which encyst has been linked by other researchers (Stevenson and Socolofsky, 1966) to the intracellular level of PHB. Thus, it would seem that an accumulation of PHB and a lack of reduced nitrogen in the medium are necessary for encystment. These two conditions may be interrelated. Indeed, the accumulation of PHB and a lack of available carbohydrate may be two preconditions for encystment in Azotobacter. Nitrogen-free medium can no longer be said to be a precondition for encystment.

It appears from microscopic observation and the staining of 96-hour-old cells with Azotobacter cyst stain that the process of abortive encystment is the normal state of over 90% of cells in Mod IV or Mod V medium after the end of log phase growth. It would appear that unless carbohydrate was held to a low level, which is not the case in most microbiological media, that abortive encystment is the common state of most Azotobacter cells during late stationary phase in a laboratory situation, and that normal encystment is a rare event. In soil, the natural habitat of Azotobacter, carbohydrate is more likely to be limiting. Therefore, abortive encystment may be the normal state of older azotobacters in most laboratory situations, but encystment may be much more likely in natural habitats.

SUMMARY AND CONCLUSIONS

A. An acceptable fixed nitrogen source for the fermentation has been described, allowing higher yields of PHB and cell mass per liter of medium than was the case if no fixed nitrogen source was supplied, or if a reduced nitrogen source was supplied. The use of nitrate in a medium for the production of PHB by Azotobacter, which has not previously been reported, results in PHB accumulations similar to those seen when no fixed nitrogen source was provided. The production of PHB when nitrate was supplied forces a re-evaluation of the conditions under which Azotobacter spp. accumulate PHB. It is evident that PHB may be accumulated in large quantities in the presence of nitrate nitrogen as well as under conditions favoring nitrogen fixation. Nitrogen fixation is not necessary for PHB accumulation in this genus.

B. It has been demonstrated that Azotobacter cells will encyst even in the presence of nitrate if excess glucose is not present. This allows a clarification of the conditions required for cell encystment in Azotobacter. Nitrogen-limitation is not a precondition for encystment. Rather, a limitation of carbohydrate and reduced nitrogen levels are necessary. The limitation of reduced nitrogen may function to allow the accumulation of PHB, which appears to be necessary for encystment.

C. Nitrogenase defective mutants of Azotobacter did not accumulate PHB, regardless of nitrogen source or carbohydrate source. This lack of PHB accumulation in cells possessing defective nitrogenase systems was not reversed, even in a nitrogenase-producing revertant.

D. High PHB-production, amylase-positive members of the genus Azotobacter were isolated.

E. The growth characteristics, nutrients, supplements, and physical parameters for optimum PHB production in the highest producer have been determined. This optimized fermentation resulted in more PHB per unit of production medium than any other published means for obtaining PHB. Several carbohydrate sources may be used with the medium and most growth supplements and minerals can be supplied by tap water and molasses.

F. Three reduced-capsule mutants were isolated. PHB production was not increased in these mutants compared with the parent strain.

G. The fermentation proceeded at a temperature slightly higher than previously reported for Azotobacter.

H. A new and superior stain for PHB was developed.

I. A new method of mass screening for PHB producers was developed.

J. The effect of phenylacetic acid on azotobacters was disproven under the fermentation conditions.

K. A superior (less involved) method of PHB extraction was developed. Many of the reagents can be recovered and reused. This method gives higher yields of PHB than the methods of Nuti, Fukui, or Wilkinson, and the method published in the Manual of Methods for General Bacteriology.

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